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BRINGING ORDER TO DISORDER – STRUCTURAL AND FUNCTIONAL ANALYSIS FOR DEVELOPMENT OF STRATEGIES TO TARGET MYC

Inga Müller

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Bringing order into disorder- structural and functional analysis for development of strategies to target MYC
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By

Inga Müller

Principal Supervisor:
Professor Marie Arsenian Henriksson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Co-supervisor(s):
Associate Professor Adnane Achour
Karolinska Institutet
Department of *Medicine Solna and Science for Life Laboratory*

Professor Mats Wahlgren
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Opponent:
Professor Linda J.Z. Penn
University of Toronto
Department of Medical Biophysics

Examination Board:
Associate Professor Sonia Laín
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Professor Anthony Wright
Karolinska Institutet
Department of Laboratory Medicine (LABMED)

Professor Ruth Palmer
Umeå University
Department of Molecular Biology

To my family

ABSTRACT

Several cellular processes such as cell cycle progression, apoptosis, cell adhesion and motility, cell growth, angiogenesis and differentiation are regulated by the *MYC* (here *MYCN* and *c-MYC*) proto-oncogenes. Elevated expression of *MYC* is present in a wide range of human tumors including Burkitt´s Lymphoma, neuroblastoma, medulloblastoma, colon cancer and glioblastoma. In cancer, high *MYC* expression levels have been correlated with fast tumor growth and poor prognosis. Neuroblastoma, a heterogeneous childhood cancer has a broad clinical spectrum ranging from spontaneously regressing tumors to those with fast progression and an unfavorable outcome. One of the markers for poor survival is *MYCN*-amplification. Importantly, down-regulation of MYCN results in differentiation. While better treatment options has increased the overall survival for low-risk tumors, the survival of children with high-risk tumors has not improved and new therapeutic strategies such as targeting MYC are needed. Currently, there are no drugs directly targeting MYC available in the clinic.

In **paper I** we demonstrated that several known c-MYC inhibitors also bind directly to MYCN and prevented MYCN/MAX interaction. We found that the affinities of the molecules correlated with growth inhibition of neuroblastoma cells. We further showed that the compounds lead to increased apoptosis in *MYCN*-amplified cells compared to non-amplified cells. In addition, treatment resulted in differentiation and lipid accumulation processes previously correlated to down-regulation of MYCN. These data show the proof-of-principle of directly targeting MYC. However, there is a great need for more potent and specific molecules targeting MYC. In **paper II** we have screened for new molecules targeting both MYCN and c-MYC. Using a cell-based assay we identified six compounds that target both MYC proteins. The small molecule #2.7 was chosen for further characterization due to its ability to directly targeting MYC. Treatment of *MYCN*-amplified and *MYCN*-non-amplified neuroblastoma cells with compound #2.7 lead to decreased proliferation in a MYCN-dependent manner and to the inhibition of transformation by RAS and MYCN *in vitro*. Collectively, our results in **paper I** and **II** support the idea that direct targeting of MYC is a new strategy for cancer treatment.

In **paper III** we demonstrated that MYCN upregulates the miR-17~92 cluster in neuroblastoma. Importantly, we showed that two members of the cluster miR-18a and miR-19a bind to and down-regulate the *ESR1* gene encoding for the estrogen receptor-α (ER-α) thereby inhibiting differentiation of neuroblastoma cells. In contrast, ERα overexpression lead to neural differentiation. We further demonstrated *ESR1* expression during normal development of the human sympathetic nervous system and showed a correlation between low ESR1 levels and poor survival. Based on these results, we suggest that targeting MYCN regulated miRNAs could be another strategy to treat *MYCN*-amplified neuroblastoma.

The transactivation domain (TAD) of c-MYC is a highly flexible region, which is necessary for all of MYC's functions. In order to gain more information about the c-MYC-TAD we have performed structural studies in **paper IV**. Circular Dichroism confirmed the presence of little secondary structure (about 9% α-helical and 30% β-sheet) in the mainly disordered (61%) c-MYC-TAD. Small-angle light scattering analysis suggested that the overall structure is most probably that of an elliptical cylinder. Our results show that small-angle light scattering together with NMR and CD might be used in future studies to gain even more insight in the structure of the c-MYC-TAD.

Taken together, two approaches to target *MYCN*-amplified neuroblastoma can be suggested based on the data presented in this thesis. The MYCN protein could either be targeted directly with small molecules or indirectly by using anti-miRNAs. We have identified several novel small molecules that target MYC that will be characterized and may have potential for further development. Importantly, we have also aimed to increase the knowledge concerning the structure of the c-MYC transactivation domain. This new information may contribute to the development of future tailored and selective targeting of MYC and its interaction partners and may also give further insight to MYC function.

LIST OF SCIENTIFIC PAPERS

I. **Inga Müller**, Karin Larsson, Anna Frenzel, Ganna Oliynyk, Hanna Zirath, Edward V. Prochownik, Nicholas J. Westwood and Marie Arsenian Henriksson.
Targeting of the MYCN protein with small molecule c-MYC inhibitors.
PLoS One. 2014;9(5):e97285

II. Karin Ridderstråle*, Qinzi Yan*, Alina Castell, Per Hydbring, **Inga Müller**, Siti Mariam Zakaria, Camilla Mannich Uggla, Marie Arsenian Henriksson and Lars-Gunnar Larsson.
Identification of low molecular weight compounds that target the MYC:MAX protein interaction and inhibit MYC-dependent tumor cell growth.
Manuscript, 2014.
*Equal contribution

III. Jakob Lovén, Nikolay Zinin, Therese Wahlström, **Inga Müller**, Petter Brodin, Erik Fredlund, Ulf Ribacke, Andor Pivarcsi, Sven Påhlman and Marie Henriksson.
MYCN-regulated microRNAs repress estrogen receptor-α (ESR1) expression and neuronal differentiation in human neuroblastoma.
Proceeding of the National Academy of Science (PNAS), 2010,107(4):1553-1558.

IV. **Inga Müller**, Ranjana Sarma, Arnaud Chêne, Eva Allerbring, Hannes Uchtenhagen, Elisabeth Kremmer, Bernhard Lüscher, Per-Åke Nygren, Tatyana Sandalova, Marie Arsenian Henriksson* and Adnane Achour*.
Structural analysis of the c-MYC transactivation domain reveals highly flexible regions in an extended rod like structure.
Manuscript, 2014.
*Equal contribution

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LIST OF ABBREVIATIONS

3´UTR	3´untranslated region
AKT/PKB	Protein kinase B
ALK	Anaplastic Lymphoma Receptor Tyrosine Kinase
APC	Activated protein C
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
BET	Bromodomain and extraterminal
bHLHZip	Basic helix-loop-helix leucine zipper
BIN-1	Bridging Integrator 1
BL	Burkitt´s Lymphoma
bp	Base pair
BRCA	breast cancer suceptibility genes
BRD4	Bromodomain containing 4
CBP	CREB binding protein
CDK	Cycline dependent kinase
ChIP	Chromatin immunoprecipitation
CD	Circular Dichroism
CSC	Cancer stem cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E-box	Enhancer box
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER-α	Estrogen receptor alpha (protein)
ERB	Epidermal growth factor
ERBR	Epidermal growth factor receptor
ESR1	Estrogen receptor alpha (gene)
FADD	FAS-Associated protein with Death Domain
FASL	FAS ligand
FBW7	F-box WD-40 domain protein 7
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GCN5	General control nondepressible-related
GRB2	Growth factor receptor bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HIF-1	Hypoxia inducible factor 1
IDP	Intrinsically disordered protein
Ig	Immunoglobulin
IGF	Insulin growth factors
INK4	Inhibitor of CDK 4

INR	Inhibitor
isPLA	*in situ* Proximity Ligation Assay
JNK	c-Jun N-terminal kinase
LDHA	Lactate dehydrogenase A
LNA	Locked nucleic acid
MAPK	Mitogen-activated protein kinase kinase
MAX	MYC Associated Factor X
MB	MYC box
MGA	MAX associated gene
miRNA	microRNA
MIZ-1	MYC interacting zinc finger protein-1
MNA	MYCN amplified
MNT	MAX´s next tango
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MYCN	v-myc avian myelocytomatosis viral oncogene homolog, neuroblastoma derived
MYCL	v-myc avian myelocytomatosis viral oncogene homolog, small lung carcinoma derived
NB	Neuroblastoma
ncRNA	Noncoding RNA
NGF	Nerve growth factor
NLS	Nuclear localization domain
O-GlcNAc	O-linked N-acetylglucosamine
ODC	Ornithine decarboxylase
PI3K	Phosphatidylinositol-3 kinase
PIKK	Phosphatidylinositol 3-kinase-related kinase
PPI	Protein-protein interactions
pRB	Retinablastoma protein
pTEFb	Postitive transcription elongation factor b
PTEN	Phosphatase and tensin homolog
qPCR	quantitative Polymerase Chain Reaction
RAS	A protein superfamily of small GTPases
RIP	Receptor interacting protein
RNA	Ribonuleic acid
RNA Pol	RNA Polymerase
SAXS	Small-angle X-ray scattering
SCF	SKP1-CUL1-F-box protein
shRNA	Short hairpin RNA
SKP2	S-phase kinase associated protein 2
SMG-1	Suppressor of morphogenesis in genitalia-1
SP1	Specificity protein-1
SUMO	Small ubiquitin-like modifier
TAD	Transcriptional activation domain

TBP	TATA binding protein
TF	Transcription factor
TGF-β	Transforming growth factor β
TRRAP	Transformation/Transcription domain-Associated Protein
wt	Wild type
Zip	Zipper

1. INTRODUCTION

1.1 CANCER

Cancer is a very complex and heterogeneous disease, in which cells divide uncontrollably leading to either benign or malignant tumors. Benign tumors do not metastasize, but the cell mass might cause local problems by damaging the surrounding tissue or by becoming malignant throughout progression of the disease [1-3]. Malignant tumors can invade nearby or distant tissues through the blood or lymphatic system and they can form metastasis. Through the last decades it has been shown that tumorigenesis is a multistep progress driven by genetic (mutations, chromosomal translocations, gene deletions, insertions or amplifications) and epigenetic alterations (chromosomal architecture, histone modifications or DNA methylations), which are either caused by environmental factors or are inherited (Figure 1) [3].

In order for a genetically altered cell to develop into a cancer cell several control systems within the cell need to be overcome. The alteration could then lead to limitless proliferation, activation of invasion and metastasis, insensitivity to anti-growth signals, sustained angiogenesis, evasion of apoptosis and self-sufficiency in growth signals [4, 5]. These mechanisms are hallmarks for developing tumors. Genomic instability and tumor promoting inflammation are supporting factors for developing cancer cells. In addition two more hallmarks for cancer have been emerging over the last years including deregulation of cellular metabolism as well as avoiding the destruction of the cancer cell by the immune system [4, 5].

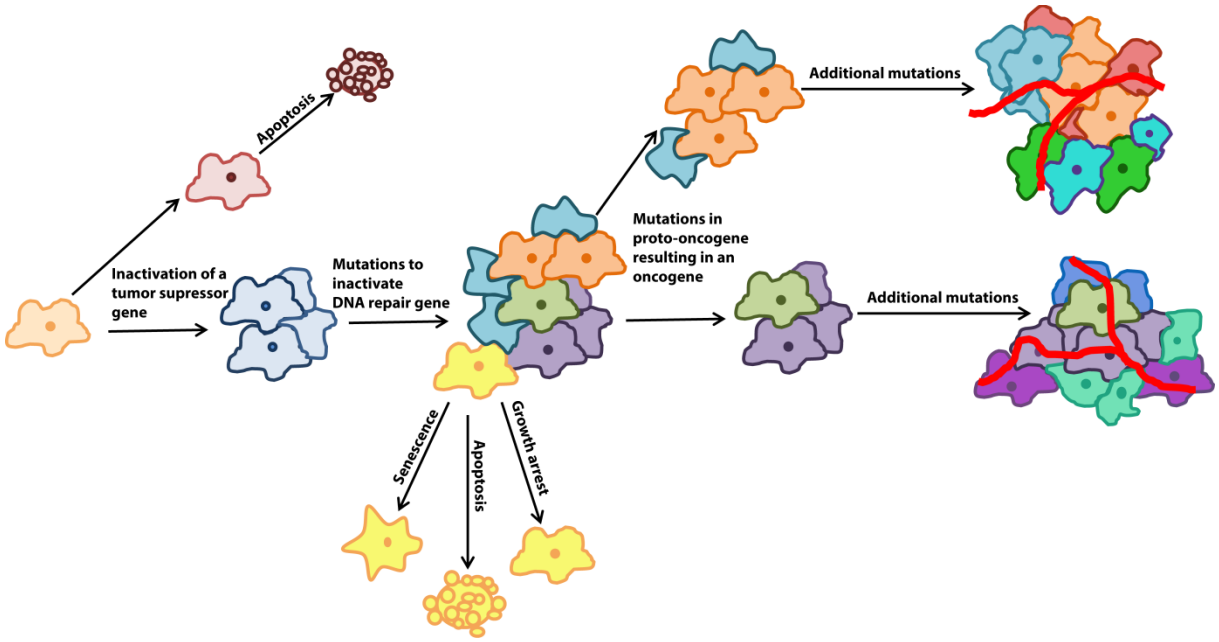

Figure 1. Possible model for the development of cancer. *After the inactivation of a tumor suppressor in a cell leading to increased proliferation, genes involved in DNA repair are mutated. This is followed by mutations in oncogenes and further mutations in oncogenes or tumor suppressors leading to the development of cancer. Each new alteration can result in an advantage in e.g cell growth or proliferation for the cell. However, there are also disadvantages alterations for the cell leading to growth arrest and apoptosis.*

Each cancer is a very unique and elaborated system, consisting of individual mutations and genetic or epigenetic alterations as mentioned above [1]. In addition, the tumor does not only consist of cancer cells but also contains normal cells such as fibroblasts, blood vessels, immune and/or stromal cells, which are part of the microenvironment of the tumor and involved in tumorigenic process [6, 7]. Cellular functions are tightly regulated by oncogenes and tumor suppressors which are deregulated in cancers [8].The function of oncogenes and tumor suppressors is explained in the next two chapters below. As mentioned above several mutations in both oncogenes and tumor suppressors are necessary in order to transform a normal cell into a cancer cell. Since these mutations do not all occur at the same time it explains the increasing incident of cancer with increasing age. However, cancer can also arise in children where they have less genetic alterations. The tumors in children originate from progenitor cells that are part of the developmental programs. In order for the pre- and postnatal development cellular processes like proliferation and apoptosis regulate and form tissues within the child. The highly proliferative cells in developing tissue together with genetic alterations in these processes can lead to cancer [9].

1.1.1. Oncogenes

In normal cells the so called proto-oncogenes are involved in normal physiological processes including proliferation, cell growth, differentiation and/or apoptosis. The products of these genes are often transcription factors, chromatin remodelers, growth factor receptors, growth factors, signal transducers and/or regulators of cell death (Figure 2) [10].

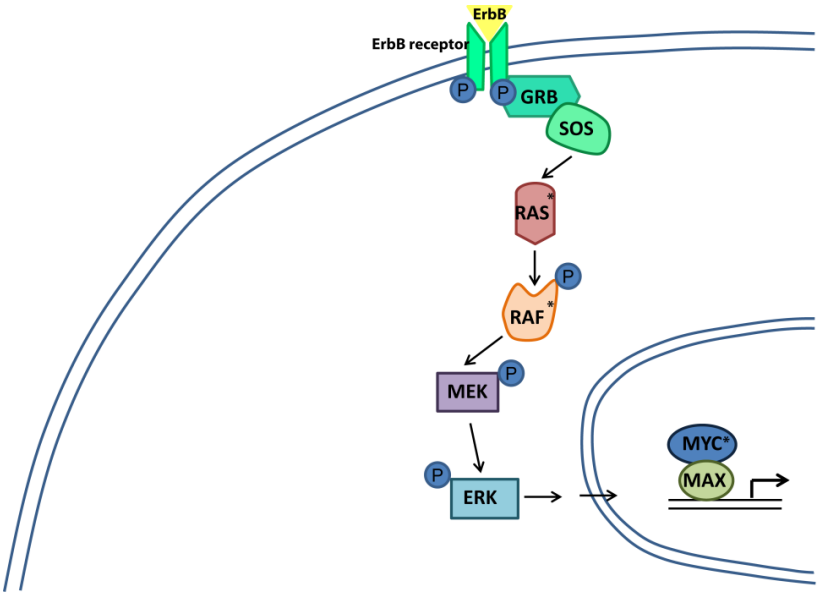

Figure 2. Examples of known oncogenes (*) in the signaling cascade. *Growth factors e.g. ErbB are binding to their tyronsine kinase receptor, e.g. ErbB receptor, which then gets activated through phosphorylation. The phosphotyrosine residues are then bound by Growth factor receptor-bound protein 2 (GRB), which in turn activates guanine nucleotide exchange factor (SOS), which in turn then activates RAS. Afterwards RAF is activated by RAS through phosphorylation, which in turn phosphorylates MEK leading to phosphorylated ERK. ERK then phosphorylates transcription factors e.g. MYC leading to their activation.*

Due to their involvement in these cellular processes proto-oncogenes can be turned into oncogenes. Alterations of the oncogene can occur through chromosomal translocation resulting in overexpression of the gene, mutations or gene amplification leading to an increased or deregulated expression of the gene. Furthermore, mutations in the oncogene can lead to a structural change of the oncoprotein, leading e.g. to decreased degradation. These alterations lead to cells with advantages to normal cells promoting cell growth and proliferation and/or pro-survival signaling in these cells [10, 11]. The proteins encoded by these genes often have multiple functions within a complex network. In their deregulated form oncogenes might be more important for the cancer cell compared to a normal cell. This might be due to fact that the gene expression and the signal transduction of the cancer cell has been shown to be different compared to a normal cell.

The first oncogenes identified in human cancer cells were the *RAS* genes, coding for small GTPases, which are involved in cellular signal transduction through e.g. the mitogen-activated protein kinases. The *RAS* genes often carry point mutations or they are amplified in wide range of human cancers such as pancreatic cancer, lung cancer and melanoma [12, 13]. Another family of oncogenes, called *MYC*, is deregulated in a large number of human tumors. This family of oncogenes encodes for the transcription factors c-MYC, MYCN and MYCL, which will be described in more detail below. In Burkitt's Lymphoma (BL) *c-MYC* is translocated to one of the *Ig* loci, while *c-MYC* is amplified in e.g. breast cancer, colon cancer and gastric cancer, *MYCN* is amplified in neuroblastoma. All these alterations lead to the overexpression of *MYC* promoting cell growth and proliferation [14]. The ALL1 protein is part of a multiprotein complex that remodels, acetylates, deacetylates or methylates nucleosomes and free histones. In acute lymphopatic leukemia (ALL) the gene encoding for *ALL1* can fuse with over 50 different genes leading to chimeric proteins, which in turn deregulate homeobox and microRNA genes[15]. The genes of growth factors like fibroblast growth factors (FGF) or insulin growth factors (IGF) have been found overexpressed in several types of cancer leading to increased signaling. Additionally, receptors of growth factors including epidermal growth factor receptor (EGFR) have been found to be altered by e.g. deletion of their ligand binding domain leading to constitutive activation (13). BCL-2, which exerts pro-survival functions in the cell, is found to be translocated in e.g. follicular B-cell lymphoma. BCL-2 is inhibiting the pro-apoptotic BCL-2-associated protein (BAX) preventing the cell from undergoing apoptosis [16, 17]. BAX, a pro-apoptotic protein of the BCL-2 family, would in turn trigger Cytochrome c release thereby also activating caspases, which would lead to apoptosis.

1.1.2. Tumor suppressor genes

With the discovery of "proto"-oncogenes it was hypothesized that genes exist, which oppose or control functions of oncogenes and could suppress tumorigenesis. These genes are called tumor suppressor genes. In order to deactivate the recessive tumor suppressor genes both alleles

normally have to be inactivated [18, 19]. Three properties of classical tumor suppressor genes have been defined. The formation of tumors is accelerated if a germline mutation exists since then only one additional mutation in the second allele is necessary. At last, the frequent inactivation of the same gene should be evident [19].

One very well-known tumor suppressor is p53, which is deregulated in over 50% of human cancers [20, 21]. p53 is also known as the guardian of the genome since it is involved in keeping the stability of the genome e.g. activation upon DNA-damage. It is a transcription factor, which regulates downstream targets involved in cell cycle control through e.g. p21, apoptosis e.g. BAX and senescence e.g. p21 and is activated from different forms of stress including DNA damage, oncogene activation, hypoxia and irradiation [19]. The p21 protein is cycline-dependent kinase inhibitor and is therefore involved in regulation of the cell cycle. The retinoblastoma protein (RB) was the first tumor suppressor discovered, it is as p53 also involved in regulating cell cycle check points [22]. RB represses gene expression by recruiting histone deacetylases and chromatin remodeling factors. Furthermore it binds to the E2F family of transcription factors. The E2F family members are involved in the positive regulation of proliferation [23]. Another group of tumor suppressor proteins are those regulating ligand-mediated signaling pathways such as Activated protein C (APC), which interacts with β-catenin in the absence of Wnt signaling leading to the phosphorylation and degradation of β-catenin and inhibiting activation of c-MYC and cyclin-D1[24, 25]. The two breast cancer suceptibility genes (BRCA) are examples of tumor suppressors that are involved in the DNA damage response. Both play key functions in homologous recombination and non-homologous end joining [19]. Another group of tumor suppressors is involved in protein ubiquitination and turnover. The von Hippel-Lindau protein ubiquitinates HIF-α leading to its degradation by the proteasome thereby indirectly inhibiting angiogenesis and glycolysis induced by hypoxia-inducible factor 1 (HIF-1α) [26].

Even though most tumor suppressors need two alleles to be mutated in order to be inactivated, there are some exceptions like the gene encoding the CDK inhibitor $p27^{Kip1}$. In these cases one of the alleles is either haploinsufficient for tumor suppression or epigenetic silencing leads to the inactivation of one of the alleles [19]. In mice, older animals that lost one copy of the *kip1* gene develop spontaneous tumors. In addition, they have been shown to be sensitive to chemical induced carcinogenesis. Furthermore there is evidence that altering one allele of $p21^{Cip1}$, $p57^{Kip2}$ and $p18^{Ink4c}$ might be enough to inactivate the tumor suppressive function of these genes [19, 27].

1.1.3. MicroRNAs in cancer

MicroRNAs (miRNAs) were first discovered as a large group of noncoding RNAs, which in most cases inhibit the translation and stability of messenger RNAs (mRNAs). Their target genes

are involved in wide range of cellular processes including cell cycle regulation, differentiation, apoptosis, metabolism, migration and stress responses [28-30].

Over 50% of all miRNA genes are clustered and transcribed as multicistronic RNA transcripts. After processing of the multicistronic RNA transcript, a single miRNA consists of a single-stranded 19-25 nucleotide (nt) long ncRNA sequence, which is now able to bind to the 3' UTR region of the targeted mRNAs resulting in the degradation and reduction of translation of these mRNAs. Each miRNA is predicted to target over 100 mRNAs and to contribute to the complexity each mRNA can be targeted by several different miRNAs [28-31].

MiRNA expression is often deregulated in cancer as a result of chromosomal rearrangements, amplification, mutations or deletions, which mostly leads to their repression compared to the levels in normal tissue. The expression of miRNAs is very tissue specific and therefore they can act as oncogene in one tissue and as tumor suppressors in other tissues [30]. One example is the*miR-17-92* cluster, which is oncogenic in several tumor types including neuroblastoma [32] but functions as a tumor suppressor in prostate cancer [30, 33]. Another example is *miR-222*, which targets the *KIT* oncogene in erythroblastic leukemia and the *PTEN* tumor suppressor in lung and liver cancer [30, 33].

1.2. PROTEIN STRUCTURE

In cells a great variety of proteins exists, which all differ in their amino acid (aa) sequences (primary structure). Understanding the folding of this linear sequence into first secondary structure elements followed by tertiary or even quaternary structure is not completely understood and has been the focus of many groups during the recent decades [34].

The α-helix and the β-sheet are the most common secondary structure elements, which are often connected by turns or loose flexible loops. AAs can be divided in different groups according to the properties of their side chains, which are: nonpolar, polar, aromatic positively or negatively charged. These properties combined with their size have been correlated with a preference for a certain type of secondary structure. While amino acids containing an aromatic ring (phenylalanine, thryptophan, tyrosine) or a bulky side chain (isoleucine, threonine, valine) are more common in β-sheets [35], short polar side chains (aspartic acid, asparagine, serine) , with no side chain (glycine) or with a cyclized side chain (proline) are preferably found in reverse turns. Except of arginine, which seems to have no preference, all other amino acids (alanine, cystein, glutamine acid, histidine, lysine, leucine, methionine, glutamine) favor α- helices [36, 37]. However, the protein fold is not only dependent on the amino acids but also on inter-residue interactions including hydrophobic, aromatic or polar contacts[38]. Furthermore, rules can be found for the distribution of residues within a certain secondary structure [38]. The properties of the solution like pH, salt concentration or the temperature play a role for secondary structure [39].

1.2.1. Globular proteins

The tertiary structure of a globular protein has hydrophobic amino acids in its core and hydrophilic, charged amino acids at the surface of the protein. Small globular proteins can fold by self-assembly [40]. Large globular proteins normally consist of several domains [41]. However it is still not clear how only the amino acid sequence can result in a three-dimensional structure [42, 43]. For a long time it was thought that proteins adapt their tertiary structure through a number of partially structured intermediates, but the chymotrypsin inhibitor 2 protein was shown to fold in a two-state transition without intermediates[44]. For the intermediates of some proteins stabilizations by chaperones is necessary during the folding process [41]. The folded, native conformation of the protein is normally the lowest free energy state.

1.2.2. Intrinsically disordered proteins

Besides completely folded proteins and multidomain proteins that are separated by flexible linkers completely intrinsically disordered proteins (IDPs) exist. During the last years it has become clear that IDPs are a very broad class of proteins with different types of functions including transcription factors and proteins involved in cellular signal transduction, protein phosphorylation, regulation of self-assembly of large multiprotein complexes and the storage of small molecules [45-48]. IDPs normally have a small content of hydrophobic amino acids, which in folded globular proteins built the inner core of the protein. Instead the proportion of charged and polar amino acids is high [45, 47]. Structural characteristics of IDPs can further be divided in extended or compact disordered state with molten globule like properties. Furthermore, IDPs involved in transcriptional activation could be divided in classes according to their aa composition. These proteins contain often acidic, proline-rich or glutamine- rich activation domains [49, 50].

Proteins with molten globule properties have been described as part of the folding of a protein gaining an ordered structure and it has not been seen as a normal state of a protein within the cell. Molten globule proteins contain native like secondary structure with little structured segments and little to no tertiary structure. Since IDPs often have several binding partners their flexibility allows for adaptation to the size and structure of their interaction partners, leading to different conformations of the IDP itself [51, 52]. Another advantage of the disordered state is the larger radius, in which it can capture its binding partners. During the binding process many IDPs gain secondary and/or tertiary structure and undergo a folding-upon binding process. The flexibility of an IDP also allows wrapping around the binding partner. The transition from disordered to an ordered associated with entropic costs that have to be balanced through a favorable enthalpy. Folding-upon-binding results in complexes that normally have a low affinity but a very high specificity leading to two different advantages [49, 50, 52]. Transcription factors can easily undergo post-translational modifications, which can modulate the protein for

its conformational requirements to bind their target protein. Such modifications are performed by phosphatases, acetylases, deacetylases, kinases, methylases as well as ubiquitin ligases. Increased numbers of phosphorylation sites have been reported for IDPs. For IDPs involved in a signaling pathway, the complex will easily dissociate after the signaling is complete. However, some IDPs remain in their disordered state after binding to their binding partner [49, 50, 52].

In order to study IDPs biophysical methods such as circular dichroism (CD), fluorescence spectroscopy, NMR spectroscopy, Raman spectroscopy and small angle X- ray scattering (SAXS) can be used. X-ray crystallography is mostly used for IDPs bound to one of their ligands. These methods are described below in the experimental methods section.

1.2.3. Transcription and transcription activation domains

Transcription is achieved by three different RNA polymerases in eukaryotes. While RNA polymerase I (Pol I) transcribes genes for ribosomal RNA (rRNA), messenger RNA (mRNA) coding for proteins and/or miRNAs and small nuclear RNA (snRNA) are transcribed by RNA polymerase II (Pol II). The third RNA polymerase (Pol III) transcribes 5S rRNA and transfer RNA (tRNA) [53]. For RNA Pol II to transcribe genes several transcription factors are needed in order to form the transcription initiation complex such as the Transcription factor II D (TFIID) complex consisting of the TATA-box binding protein (TBP) and TBP- associated factors (TAFs) [53]. The TATA-box sequence that is located some base pairs upstream the initiation site, is recognized by TBP followed by recruitment and association of additional transcription factors (TFIIA, TFIIB, TFIIE, TFIIF and TFIIH) [54]. After the DNA double strand is separated and Pol II has bound to the initiation complex, RNA is synthesized [53].

In order to regulate gene expression by Pol II complex, DNA regulatory sequences are bound by transcriptional activators and repressors. This mechanism allows genes to be turned on or off. Proteins involved in activation or repression often have several domains that are connected by flexible linkers. The most important domains for these proteins are a DNA binding domain and the transcriptional activation domain (TAD), which is often bound by a variety of cofactors, repressors and proteins of the transcriptional complex. Even though the biological functions of TADs have been studied, much remains unclear regarding their structure and the molecular mechanism of interaction. There is no real sequence homology between different TADs and due to their disordered state and multiple binding partners their structures are not easy to assess. TADs have been proposed to interact with their binding partners through short hydrophobic stretches [55, 56].

1.3. THE MYC FAMILY

After the identification of the human homologue of the *v-gag-myc* gene in Burkitt's Lymphoma (BL) in the early 1980s [*cellular MYC (c-MYC)]*, two more genes called *MYCN* and *MYCL* were discovered. The name MYC is derived from **my**elo**c**ytomatosis. While *MYCN* was found to be amplified in neuroblastoma *MYCL* was identified in small lung carcinoma[57, 58]. *MYC* is involved in a number of cellular processes such as apoptosis, proliferation, cell growth, inhibition of differentiation, metabolism, telomerase activity, cell adhesion and motility as well as angiogenesis. Deregulation of *MYC* can lead to uncontrolled cell proliferation, immortalization, independence of growth factors, escape from immune surveillance and genomic instability. Both normal functions as well as results of deregulated *MYC* will be described below [14, 58, 59].

1.3.1 MYC expression and regulation

In general *c-MYC* mRNA is expressed at low levels and also present in normal proliferating cells [60]. A tight regulation on multiple levels such as transcription initiation, transcriptional elongation, translation, stability of mRNA has been shown. For example it has been shown that the H3-histones surrounding the *c-MYC* gene are significantly more hyperacetylated, while no change in the levels of H3-H9-methylation could be found [60]. In 1986, *c-MYC* was the first gene identified to be regulated by transcriptional elongation control [61].

There are actually four *c-MYC* promoters, but the level of initiation is very low for the P0 and P3 promoters, since they lack the TATA-box binding motif. The TATA-box binding motif of the P1 Promoter is less optimal and transcription is started with up to 25% at this starting site. The P2 promoter contains an optimal TATA-box with two additional initiator (INR) elements, which explains why this promoter is used for transcriptional initiation in up to 90 %. There are two different proteins produced, the larger one with a size of 67 kDa from a start codon in the end of the first exon and a smaller one with 64 kDa, which is the main protein product from an ATG start codon in the second exon [60, 62].

During gastrulation *c-MYC* and *MYCN* are expressed in contrasting patterns [63]. Both genes are differentially expressed in all types of tissues during embryonic development. While *MYCN* expression is restricted to the developing kidney, intestine, brain, lung and heart and high expression has also been found in the neuroepithelium, *c-MYC* is expressed in most tissues [64-66]. *MYCL* expression is restricted to certain tissues e.g. fetal skin and specific stages of development. Knockout mice of the *c-myc* gene die at embryonic day 10.5, in contrast *mycn* knockout mice die at embryonic day 11.5 [67, 68]. In *c-myc* null mice an introduction of the *mycn* gene in the *c-myc* locus resulted in viable mice with only few developmental defects in the exception of skeletal muscles[69]. In the adult *c-myc* is expressed in nearly all tissues, while *mycn*

is very low or absent [70]. *MYCL* is expressed in the lung in the adult. The levels of the *MYC* genes are low in non-dividing cells, but the response to growth stimuli is fast and the expression of the gene is rapidly elevated. The expression of *c-MYC* correlates strongly with cell proliferation and levels of *c-MYC* in the nucleus are constant throughout the whole cell cycle [71, 72]. *MYC* genes underlie high levels of control and in response to anti-proliferative signals the *MYC* gene is immediately down-regulated if the cell is not proliferating. In case the gene would not be down-regulated after anti-growth stimuli, the cell would undergo apoptosis due to regulatory mechanisms in the cell measuring MYC-levels [73, 74].

1.3.2. MYC protein

MYC consists of the N-terminal transactivation domain (TAD), the central domain and the C-terminal basic helix loop helix leucine Zipper domain (bHLHZip) that is able to bind to DNA. The C-terminal bHLHZip domain as well as MYC box I and II (MB) in the TAD are very conserved regions (Figure 3). In addition there are two more MYC boxes (MB III and MB IV), which are conserved in c-MYC and MYCN and c-MYC, MYCN and LMYC respectively [58, 75, 76].

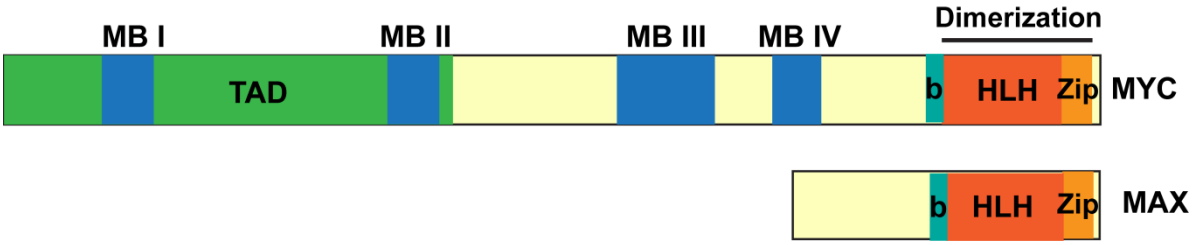

Figure 3. Schematic overview of the MYC and MAX proteins. *The MYC protein contains several conserved domains. The N-terminal transactivation domain harbors two conserved regions MYC-box (MB) I and II. MB III and IV are located within the central domain, while the C-terminal region consists of a basic helix-loop-helix Zipper domain (bHLHZip). The MAX protein also contains a bHLHZip domain.*

The c-MYC TAD consists of the residues 1-143 and contains MB I (44-63) and MB II (128-143). MB I is involved in gene activation as well as the degradation of MYC by phosphorylation of T58 and S62. The deletion of MB I does not have major effects on MYC function. Hot spots for mutations have been found in MB I at residues T58 and S62 as well as the surrounding sequence in BL [58, 77]. In contrast to MB I, MB II is crucial for most of MYCs functions and the deletion of MB II leads to a strong reduction of its transcriptional activation and transformation capacity. In MB II E138 has been identified as a hot spot for mutations in BL [58]. Two domains with transactivating activity have been localized. The first domain is situated next to MB I comprising residues 1-41. The second domain is overlapping MB II involving residues 66-127. Also two domains for transcriptional repression have been localized.

While one domain is overlapping MB II the second domain is localized between MB I and MB II (residues 92-106) [78]. Recently it has been shown that MB III, which is located in the central domain, is involved in transcriptional repression and is able to negatively regulate transformation by increasing MYC's potential to induce apoptosis. Deletion of MB IV resulted in the impairment of MYC's ability to bind to DNA as well as in hyperactive G_2 arrest. Furthermore, MB IV was shown to be important for induction of apoptosis and might play some role in transformation [79]. The bHLHZip domain of MYC is heterodimerizing with Max leading to DNA binding. This domain has also been implicated in the oligomerization of MYC produced in bacteria. However, this did not seem to be a physiological mechanism. In addition, this domain is necessary for MYC's ability to cellular transformation [78].

1.3.2.1. Structure of the MYC protein

In the unbound form MYC is an IDP (see section about intrinsically disordered proteins) meaning that upon binding MYC will form secondary and eventually tertiary structure. The structure of the C-terminal bHLHZip domain of c-MYC has been determined in a heterodimer with MAX bound to DNA[80]. Upon interacting with MAX c-MYC forms two α-helices that are connected by a loop enabling the complex to bind to DNA. However, little is known about the structure of the N-terminal TAD. This domain has been shown to interact with a large number of different binding proteins, thereby probably obtaining different confirmations. Recent efforts have revealed some structural insights in parts of the c-MYC-TAD. The domain without interaction partner has been predicted to have about 20% α-helices and some β-sheet strands [81]. Indeed, the presence of short α-helical segments has been reported. Two short α-helices have been reported for the residues 26-34 and 47-52. In addition a short β-sheet strand stretching from residues 22-33 has been found [81, 82]. Predictions have further indicated a partly α-helical fold for the region comprising residues 92-167. Within this region lies the most hydropobic part, which is located in MB II [78]. Our study (**paper IV**) also indicates some α-helical structure as well as some β-sheet strands. Furthermore the SAXS analysis we performed, implicated structured domains that are connected by flexible linkers within the c-MYC-TAD. The c-MYC-TAD alone was also found to oligomerize (**paper IV**).

1.3.2.2. Post-translational modifications of MYC

Nuclear proteins involved in regulation of gene transcription are often targets of several different signal transduction pathways. Therefore these proteins are regulated at several different steps by post-translational modifications. MYC has been reported to be phosphorylated, ubiquitinated, acetylated, glycosylated and sumoylated [83].

1.3.2.2.1. Phosporylation of MYC

Several phosphorylation sites have been identified for MYC including T58, S62, S71, S82, S162 and S164 in the N-terminal TAD. Furthermore two additional clusters have been identified including residues T247, T248, S249, S250, S252 as well as T343, S344, S347 and S348. The two phosphorylation sites T58 and S62 have been well described due to their involvement in MYC degradation. Both amino acid residues are targeted by the kinase glycogen synthase kinase 3 (GSK3) for T58 as well as proline-directed kinases such as mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and cyclin-dependent kinase 1 (CDK1) for S62 phosphorylation. The different kinases phosphorylating S62 are involved in several different signaling pathways. In order to modify T58, S62 has to be phosphorylated by one of the kinases. The phosphate is then able to bind specifically in the substrate recognition domain of GSK3, enabling for phosphorylation of T58. Other phosphorylation sites such as S71, S82, S293 and S344 are also close to prolines next to them, which makes them potential substrates for proline-directed kinases. To date no kinases have been identified that might be involved in the modification of these serines.[84-86].

1.3.2.2.2. Ubiquitination and degradation of MYC

Through poly-ubiquitination of lysine residues, proteins can be targeted for degradation through the 26S proteasome. The activity of the MYC protein is tightly regulated and the protein has a short half-life of 20-30 minutes. After phosphorylation of both T58 and S62, S62 is dephosphorylated by the protein phosphatase 2A. The F-box protein FBW7 is now able to bind MYC, which is only phosphorylated at T58. FBW7 is a subunit of the SKP1-CUL1-F-box protein (SCF) ubiquitin-protein isopeptide ligase complex, which is able to poly-ubiquitinate MYC leading to its degradation. In addition, MYC can also be ubiquinated through the SCF-SKP2 complex. It has been suggested that this interaction leads to the ubiquitinylation of MYC leading first to the activation of the transcriptional activity of MYC followed by its degradation[87-91].

1.3.2.2.3. Acetylation of MYC

Several proteins binding to the c-MYC-TAD are additionally also interacting with histone acetyltransferases, which are not only able to acetylate histones but also MYC itself. Two proteins, p300 and mGCN5 are able to modify lysines (K298, K323, K326, K341 and K355) which is located within the nuclear location sequence (NLS). Other lysines within MYC such as K417 in the Zip can also be acetylated [92]. Recently, these six lysines within c-MYC were mutated to arginines leading to increased cell proliferation as well as the ability to transform cells and to enhance tumor growth in a NB and a breast cancer model [93]. The lysines in and

close to the c-MYC-TAD (K143, K148 and K157) have also been reported to be acetylated [94]. Acetylation can also interfere with ubiquitinylation since both target lysines. Through acetylation of MYC it has been show that ubiquitinylation of MYC is decreased suggesting that the MYC protein can be stabilized[95, 96].

1.3.2.2.4. Glycosylation of MYC

Serine and threonine are amino acids that can be modified by O-linked N-acetylglucosamine (O-GlcNAc), which can be linked to the hydroxyl side chain of the two amino acids. O-GlcNAcylation has been shown to play an important role during cell proliferation as well as chromatin modulation. Manipulations of O-GlcNAc levels affect the migration and or invasiveness of e.g. breast and lung cells [97]. Furthermore increased O-GlcNAcylation has been reported for several types of cancer [97]. MYC is also glycosylated close to the or within the c-MYC-TAD. T58 has been shown to be O-GlcNAcetylated and thereby MYC protein stability is increased. However, the status of MYC glycosylation still needs to be further examined [98, 99].

1.3.2.2.5. Sumoylation of MYC

During sumoylation one or several small ubiquitin-like modifier (SUMO) proteins are coupled to lysines. Higher eukaryotes have three SUMO paralogs. There are three effects of sumoylation on their target proteins, namely competition with acetylation and ubiquitinylation, creation of new interactions or inhibition of protein-protein interactions. Therefore SUMOylation plays a role in different cellular processes like transcription, DNA repair and the structure of chromosomes. It has been reported that MYC can be SUMOylated on a lysine between MB IV and the bHLHZip domain of both c-MYC and MYCN. The effects of SUMOylation are not entirely clear, however it has been suggested that it might play a role in controlling misfolded MYC proteins [100].

1.3.2.3. MYC interaction partners

During the last decades several binding partners to both the C-terminal bHLHZip domain as well as the N-terminal TAD have been identified (Figure 4). One of the best characterized interactions is the heterodimerization of c-MYC and MAX, which will be described below. For some binding partners only little is known and much remains to be investigated. Especially the molecular basis underlying binding of the MYC-TAD and its binding partners still needs to be examined. A selection of binding partners to both domains will be described below in more detail [75, 78].

1.3.2.3.1. Binding partners to the N-terminal domain of MYC

1.3.2.3.1.1. **TR**ansformation/t**R**anscription domain-**A**ssociated **P**rotein (TRRAP)

The TRRAP protein is conserved throughout many species and with 3828 amino acids it has a very large size. It is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, which is related to PI3-kinase (PIK) family [101]. The proteins of this family play a major role in DNA damage detection and signaling. In mammalians this family includes proteins such as mammalian TOR (mTOR), Ataxia-telangiectasia mutated (ATM) or the suppressor of morphogenesis in genitalia-1 (SMG-1). These proteins share several motifs, like the FATC (FRAP, ATM and C-terminus) domain, the FAT (FRAP, ATM and TRRAP) domain as well as the PIK-like domain. However TRRAP lacks the conserved aa's necessary for the catalytic activity of these proteins and therefore lacks kinase activity. Instead TRRAP might have evolved as an adaptor or scaffold for protein-protein interaction as well as for multiprotein assembly [101]. TRRAP serves as a platform for regulatory factors and protein complexes involved in the coordination of processes modulating chromatin as well as in transactivation and repression of genes. It is a very common subunit of different histone acetyltransferases (HAT) complexes like the general control nonrepressible-related (GCN5) acetyltransferases (GNAT) HAT family and the MOZ, Ybf2/Sas2, Tip60-related (MYST) HAT family [102].

TRRAP has been found to be involved in embryonic development as well as cell cycle progression [103]. The recruitment of TRRAP by c-MYC to promoters transcribed by Pol I has previously been shown[101]. Two regions in c-MYC have been reported to be bound by TRRAP. One region in c-MYC is at MB II and another region closer to the N-terminal end of the protein. The main region of TRRAP involved in this interaction has been located to a stretch spanning aa 1899-2026. Furthermore the region comprising aa residues 1261-1579 as well as residues 3402-3828 has been shown to be involved in inhibition of c-MYC-dependent transformation either through direct binding to c-MYC or by sequestration of a downstream target of the c-MYC/TRRAP pathway [103-105]. In a glioblastoma cells *TRRAP* knockdown has led to an increase in apoptosis and induction of differentiation in brain tumor initiating cells (BITC). Furthermore, a decrease in tumor burden and an increase in overall survival in a glioblastoma xenograft model. These results suggest a close involvement of TRRAP in transformation of cells and as a result tumor formation [106].

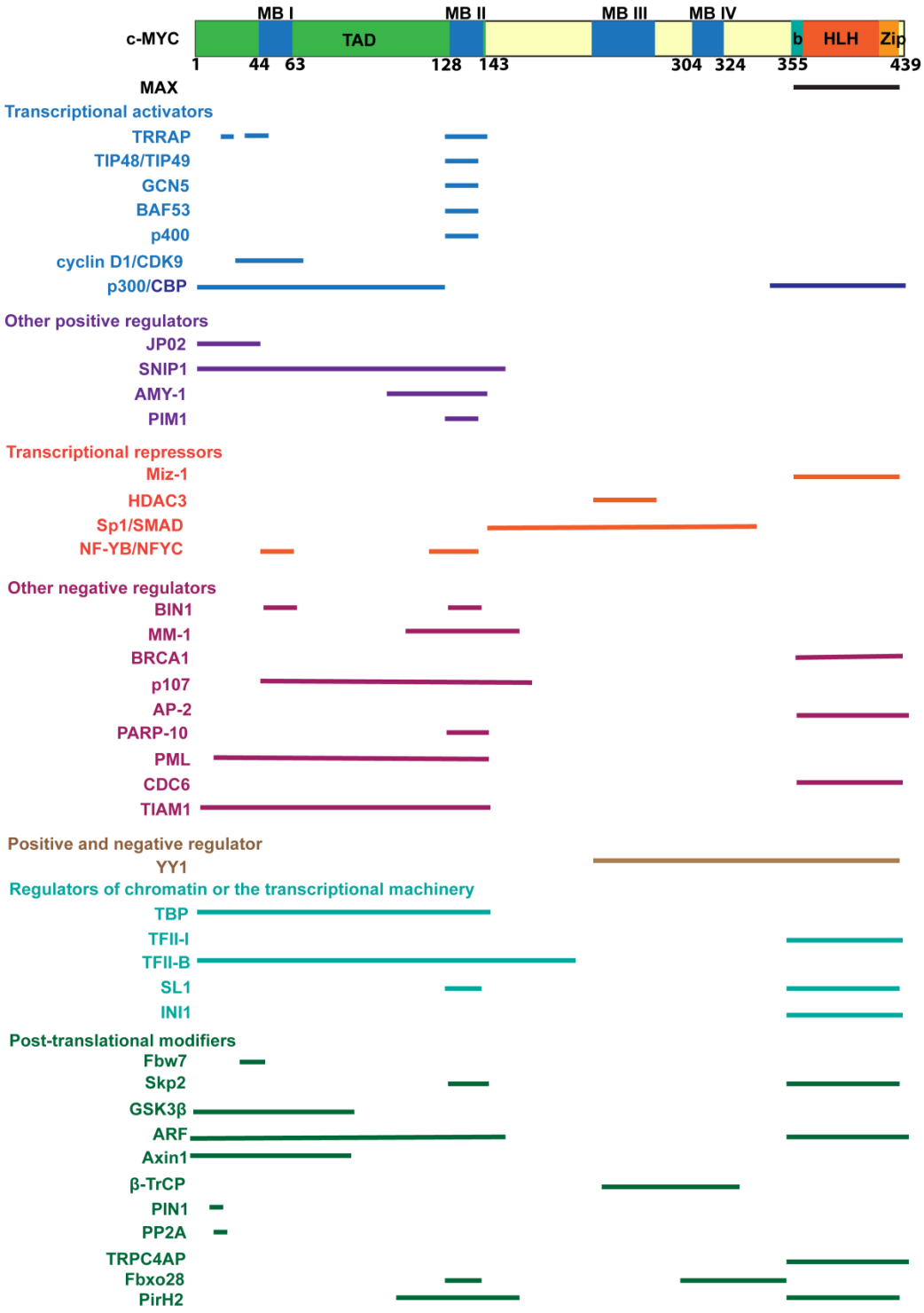

Figure 4. MYC and its binding partners. *The most important domains in MYC like the transactivation domain (TAD), the MYC-boxes (MB) I-IV as well as the basic helix-loop-helix domain (bHLHZip) are marked. Regions for interaction by MYC and their binding partners are indicated. Adapted by Tu et al. (2014), with the permission from the publisher.*

1.3.2.3.1.2. **T**ATA**B**inding **P**rotein (TBP)

TBP plays a central role in gene expression, interacting with all three RNA polymerases through the formation of complexes such as selectivity factor (SL) 1, TFIID or TFIIIB. TBP is an important subunit of the TFIID and SPT-ADA-GCN5 acetyl-transferase (SAGA) complexes, which bend and unwind DNA. TBP has been shown to bind to an extended sequence of the c-MYC-TAD. Upon binding an induction of protein conformation within the c-MYC-TAD has been reported. Furthermore TBP has been suggested as an important target for the c-MYC-TAD during transcriptional initiation [107, 108].

1.3.2.3.1.3. **B**ridging **IN**tegrator 1 (also **B**ox-dependent myc-**IN**teracting protein 1; Bin-1)

The tumor suppressor BIN-1 contains a N-terminal Bin-Amphysin-Rvs (BAR) domain and a C-terminal sarcoma (SRC) homology domain as well as a neuron-specific endocytic function domain (NTS). Furthermore interaction of BIN-1 with c-MYC through the MYC-binding domain (MBD) has been reported and selectively inhibiting MYC's oncogenic and transactivation properties. Several tissue specific splicing variants have been identified, some of which have been implicated to play a special role in tumorigenesis. This may be a result of some isoforms, which abolish the ability of BIN-1 to interact with c-MYC [109].

In a structural binding study of the aa 1-88 of c-MYC to the SH3 domain of BIN-1 no folding upon binding could be detected [81]. Deletion of the MBD resulted in loss of the ability of BIN-1 to interact with c-MYC and therefore lead to uninhibited c-MYC mediated tumorigenesis [110]. The tumor suppressing function of BIN-1 has also been shown to be exerted through a MYC-dependent and a MYC-independent mechanism like the inhibition of RAS co-transformation [110].

1.3.2.3.1.4. **F**-**B**ox and **W**D repeat domain-containing 7 (FBW7; FBXW7)

Three classes can be found for F-box proteins depending on the presence of WD40 repeats (FBXW), leucine repeats (FBXL) or the absence of both domains. The F-box proteins are adaptor proteins for SCF ubiquitin ligase complexes and substrate variation is depending on the F-box protein bound by the complex and their target proteins. FBW7 has been shown to regulate processes such as cell growth, proliferation, differentiation and survival through degradation of its target proteins such as c-MYC, cyclin E, NOTCH and c-JUN. These proteins are targeted by ubiquitinylation through FBW7 at specific residues and down-regulation of FBW7 expression or mutations impairing the function have been found in a variety of human cancers leading to the assumption of FBW7 as a tumor suppressor [89].

It has been shown that FBW7 recognizes c-MYC and is responsible for its ubiquitinylation and degradation. It has been reported that during the cell cycle FBW7 is regulating c-MYC stability in G1 phase. Recently it has been found that another ubiquitin ligase β-TrCP attaches polyubiquitin chains on c-MYC thereby antagonizing FBW7 induced c-MYC degradation [87, 89, 111].

1.3.2.3.1.5. **S**-phase **k**inase associated **p**rotein 2 (SKP2)

SKP2 is another F-box protein, which is regarded as an oncogenic protein since it degrades tumor suppressors and is involved in cell cycle progression. After gradually increased *SKP2* expression throughout the cell cycle, the highest levels of expression can be found in S-phase. In resting cells *SKP2* expression is low. It is known to degrade the CDK inhibitors p21 and p27 leading to a progressing cell cycle. Other SKP2 substrates are e.g. p57, BRCA2, RAG2, E2F1 and MYC. In several types of tumors deregulation of SKP2 has been correlated to poor prognosis. Interestingly, besides leading to the ubiquitinylation of c-MYC and its degradation, SKP2 has also been reported to promote transactivation as well as MYC mediated transformation. Interaction between SKP2 and c-MYC seems to involve MB II as well as the bHLHZip domain [91, 112, 113].

1.3.2.3.2. Binding partners to the C-terminal domain of MYC

1.3.2.3.2.1. **M**YC inter**a**cting factor **X** (MAX)

The helix-loop-helix leucine zipper protein MAX plays a central role in cellular processes and the transcriptional control of the MYC family. While MYC-MAX heterodimers positively regulate transcription, MAX homodimers or heterodimers of MAX with one of the MXD family members, MNT or MGA negatively regulate transcription of MYC target genes [114, 115] MAX is a very stable protein however the MAX-MAX homodimer is not very stable. Two major isoforms,p21 and p22, of MAX have been identified [116, 117]. The larger protein binds to DNA with higher affinity than the smaller protein. All proteins of the MYC-MAX-MXD network contain a bHLHZip domain that allows heterodimerization between MAX and one of the network members allowing binding to CACGTG-E-box sequences. The structure of the MAX homodimer bound to DNA has been determined by X-ray crystallization. It revealed an N-terminal α-helix, followed by two α-helices that are connected by a loop in the HLH domain and another α-helical coiled coil in the Zip domain [118]. NMR studies of MAX without DNA showed an unfolded basic region that is stabilized and folded upon binding to DNA. Also the Zip is partly unfolded if MAX is not bound to DNA and the rest of the C-terminal half as well as both protein ends are unstructured. While MAX is needed for most of MYC's functions recent reports have suggested that MYC is also able to perform some of its functions without

interaction with MAX [119-122]. For example, a version of MYC, called MYC-Nick, has been shown to stimulate proliferation in rat fibroblasts, probably through translational mechanisms [123, 124].

1.3.2.3.2.2. **M**YC-**I**nteracting **Z**inc finger protein 1 (Miz-1)

MIZ-1 is a zinc finger protein containing a Pox virus and Zing finger (POZ) domain. Depending on its interaction partners the MIZ-1 transcription factor is working as a transcriptional repressor or activator. It is involved in growth arrest and can normally be found in the cytosol. Furthermore it binds to initiator elements and activates the promoter of cyclin D1 and the adenovirus major late promoters [125]. An amphipathic helix in MIZ-1 is interacting with the HLH domain of c-MYC thereby inhibiting transcription of MIZ-1. Association with MYC leads to its translocation into the nucleus. Furthermore MIZ-1 has been implicated in stabilizing MYC against degradation through ubiquitinylation [126-131]. Recently it has been shown that gene repression by MYC together with MIZ-1 is dependent on the ratio between these two proteins [132].

1.4. CELLULAR FUNCTIONS OF MYC

MYC is involved in a number of cellular processes such as apoptosis, proliferation, cell growth, inhibition of differentiation, metabolism, telomerase activity, cell adhesion and motility as well as angiogenesis (Figure 5). Deregulation of *MYC* can lead to uncontrolled cell proliferation, immortalization, independence of growth factors, escape from immune surveillance and genomic instability [14, 57]. Both normal functions as well as results of deregulated *MYC* will be described below.

1.4.1. MYC in apoptosis

In order to survive a cell needs pro-survival factors (cytokines), which are bound by cell surface cytokine receptors. These receptors signal through e.g. the AKT/ protein kinase B (PKB) and protein kinase A (PKA) families of serine/threonine kinases leading to the survival of the cell. Apoptosis is a function that leads to controlled cell death, which is very important for e.g. the development of organs and tissue or after e.g. DNA damage. There are two pathways for induction of apoptosis namely the mitochondrial and the receptor pathway [133]. Several stress stimuli can lead to induction of apoptosis such as UV-light, radiation, chemotherapeutic drugs or cytokine deprivation. After activation of BAX or BAK, BCL-2 is bound by one of the proteins, diminishing the pro-survival function of BCL-2 leading to cytochrome c release as well as additional pro-apoptotic factors from the mitochondria [16, 17, 134]. This in turn leads to the

activation of caspases and to cell death . Another way to activate cell death receptor FAS is through the FAS ligand (FASL) leading to the formation of a tetramer of the FAS receptor. The FAS-Associated protein with Death Domain (FADD) and the FAS receptor itself contain a death domain DD, which is necessary for their interaction. This in turn results in the recruitment of pro-caspases, which after cleavage will initiate the caspase cascade leading to apoptosis [17, 135, 136].

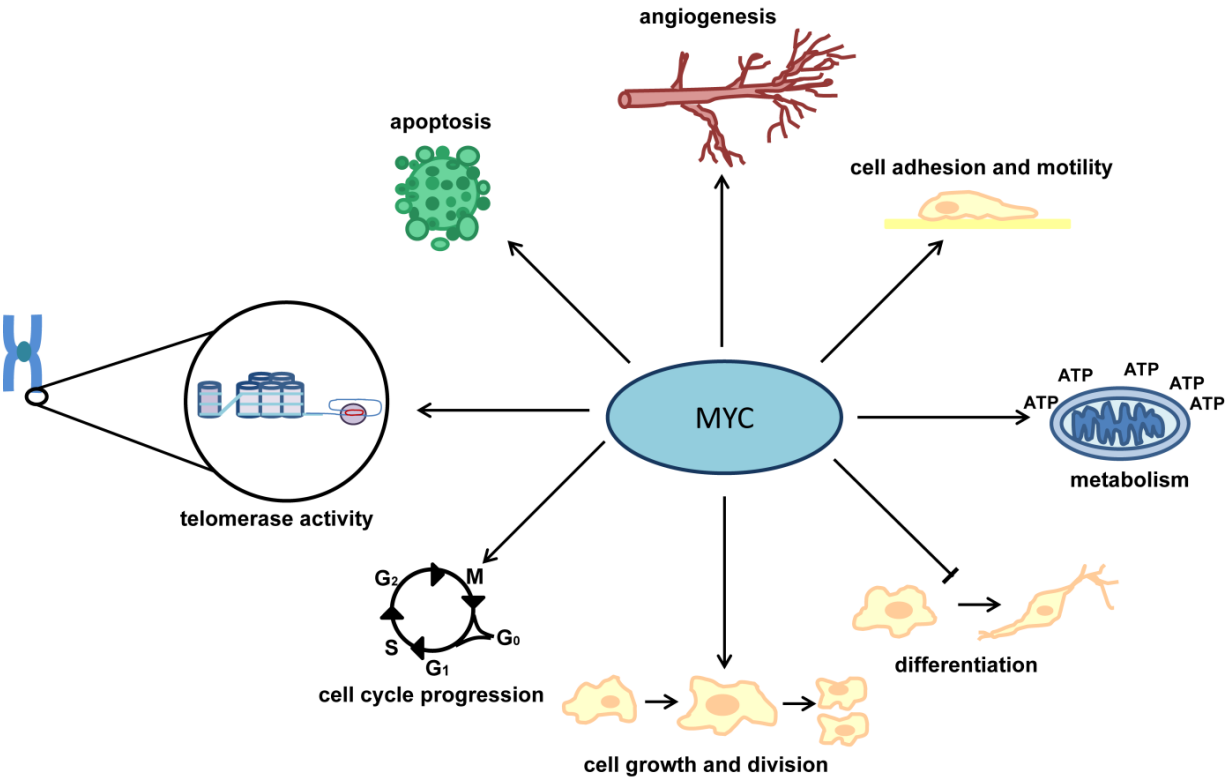

Figure 5. Functions of MYC. *The transcription factor MYC is involved in the regulation of cellular processes such as cell cycle progression, cell growth, differentiation, metabolism, cell adhesion and motility, angiogenesis, apoptosis and telomerase activity.*

High expression of *MYC* in normal cells as well as in some cancer cells sensitizes them to pro-apoptotic stimuli. Several MYC targets have been identified to play an important role in apoptosis. The death receptor pathways can be influenced by MYC on several levels. First of all MYC is able to activate the expression of FASL. This either leads to the direct suicide of the cell or in regard to MYC's function in escaping immune surveillance it can also lead to the death of cancer cell targeting immune cells expressing *FAS* [73, 137].

The serine/threonine kinase receptor interacting protein (RIP) is also activated by MYC in order to sensitize cells to tumor necrosis factor alpha (TNF-α) mediated apoptosis. In addition, an

indirect target of MYC is NF-κB and down-regulation of its activity leads also to inactivation of downstream pro-survival factors like BCL-2 [138, 139].

Two proteins of the BCL-2 (BCL-2 and BCL-X_L) family are also regulated by MYC. The proteins of the BCL-2 family have either anti-apoptotic or pro-apoptotic properties. On the one hand MYC can induce association of BID with the mitochondria probably leading to the disruption of the functions of anti-apoptotic proteins such as BCL-2 or BCL-X_L. On the other hand MYC can also indirectly inhibit the induction of the *bcl-X* and *bcl-2* genes and their respective proteins. These proteins can in turn inhibit BAX, which is needed for the progression of apoptosis [73, 137].

After the activation of MYC protein levels of ARF are increased. ARF then binds directly to Mdm2, thereby blocking its E3 ligase activity that normally leads to the inhibition of its activity as ubiquitin ligase for p53 and the sequestering of MDM2 in the nucleolus is initiated [140]. The activation of p53 in turn leads to activation of proteins like PUMA, NOXA and BAX involved in apoptosis induction as well as the cell-cycle inhibitor p21. BAX is involved in the control of cytochrome c release, which then enables the apoptosome to form as well as the activation of caspases, inducing the final step of apoptosis [141].

1.4.2. MYC in the cell cycle, cell growth and differentiation

During development as well as wound healing cell mass is accumulated in order to grow and to develop organs and tissue or to close the wound. Cells will normally not grow without getting mitogenic growth signals from the surrounding environment. The process is called cell cycle and is highly regulated. The cell cycle is divided in different phases including G_1-, S-, G_2-phase and mitosis (also M-phase), which is further divided in prophase, metaphase, anaphase and telophase. After a division a cell will either remain proliferating or become a resting non-proliferative cell in G_0-phase.This decision will be made until the restriction point (R-point) in late G_1-phase. From the beginning of G_1 until the R-point, the cell is able to respond to mitogenic growth factors. If the cell continues to proliferate progression to each phase will be monitored by checkpoints in order to avoid DNA damage [24, 142, 143].

Cyclin-dependent kinases (CDKs) together with cyclins are necessary for cell cycle progression at each checkpoint. While CDKs are stably expressed during the cell cycle, cyclins are synthesized and degraded when they are needed. In the beginning of the G_1- phase the presence of mitogenic signals are sensed by the cell and the expression of cyclins D1, D2 and D3 are upregulated, which are needed for the activity of CDK4 and CDK6. The RB protein is phosphorylated at some few residues [143]. In late state G_1-phase after activation of *cyclin E* expression, the cyclin E protein binds to CDK2 in order to activate it. RB is further phosphorylated after the R-point leading to the activation of E2F transcription factors [144, 145]. In turn expression of cyclin A1 and A2 is initiated, which are needed for S- and G_2-phase. CDK 1

together with B-cyclins is present throughout mitosis after their expression has been activated.In the end of the mitosis the hyperphosphorylated RB protein is dephosphorylated. This is essential especially for cells in G_0-phase [143].

In order to control the cell cycle, so called cyclin-dependent kinase inhibitors (CKI) can function as negative regulators. Four INK4 proteins, called $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$ are able to inhibit CDK4 and CDK6. The other four CDKs can be inhibited by $p57^{Kip2}$, $p27^{Kip1}$ and $p21^{Cip1}$. In this way the cell cycle can be inhibited at every step if *e.g.* DNA damage would occur during cell division [143].

Upregulation of *MYC* in quiescent cells is enough to initiate cell cycle entry. During the cell cycle *MYC* is expressed in all phases and its targets are also cell cycle related genes like *CDK4, cyclin D1, cyclin E1, E2F1* and *CDC25A* explaining its importance during proliferation [146]. In addition MYC is also able to induce CDK activating kinases, which lead to activation of CDK's. Furthermore MYC also represses the CDK kinase inhibitors *p21* and *p27*. While *p21* is suppressed by the MYC/Miz-1 complex, MYC represses *p27* by e.g. induction of miR-221 and miR-222, which silence the *p27* mRNA [147].

One important aspect of cell division is cell growth. A freshly divided cell needs to accumulate biomass and grow. For this process MYC is involved in several processes ribosomal and mitochondrial biogenesis and the metabolism of the cell as well as nucleic acid and protein synthesis. These mechanisms are regulated by MYC through transcriptional activation of genes involved in these processes. In ribosomal biogenesis for example MYC regulates all three RNA polymerases by interacting with their different regulatory factors. MYC is also involved in the regulation of the Transcription Factor A Mitochondrial (TFAM) protein, which is needed for mitochondrial transcription and DNA replication [148, 149].

Differentiation is a process during which cells acquire a more specialized phenotype that is more characteristic for the tissue or organ to which each cell belongs to. Down-regulation of genes involved in proliferation are necessary. Indeed it has been shown that the *MYC* gene is strongly down-regulated during differentiation. However, recently it has been demonstrated that MYC not only blocks but is also able to facilitate terminal differentiation. This has been shown in several precursor cells including neuronal cells, pre-B-cells and skin stem cells [123, 150-152]. *Eμ-myc* transgenic mice have an impaired development of their B-lymphocytes [153]. Neuronal progenitor cells could not differentiate when *MYCN* was expressed and a deletion of *MYCN* in mice induced premature differentiation of neuronal progenitor cells [154]. Additionally, the cleavage product of MYC by calpain, so called MYC-Nick, has been reported to be increased in the cytoplasm of differentiating muscle cells [123]. Calpains are activated by increased levels of calcium. In hematopoietic stem cells the overexpression of MYC leads to differentiation while *MYC* knockout reduces differentiation [123].

In murine embryonic stem cells c-MYC induces miRNAs that silence expression of genes involved in differentiation and modulating in this way the differentiation of these cells. Another way MYC is hindering differentiation is through repression of transcription factors including c-JUN and GATA-1 [155].

Reprogramming of already differentiated cells like fibroblasts into induced pluripotent stem (iPS) cells has been shown to be possible with the expression of only four transcription factors, *Oct-4*, *Sox-2*, *Klf-4* and *c-MYC*. Results from 3T3-L1 cells and mice suggested that MYC functions for inhibiting differentiation are necessary for MYC's transforming activity [156-158].

1.4.3. MYC in metabolism

The metabolism of proliferating cells consists of several different processes including glycolysis resulting in ATP production, lactate production, intermediates of the TCA cycle as precursors for lipids, proteins and nucleic acids [159-161]. Otto Warburg showed already in 1931 that the metabolism of normal non-proliferating cells differs from tumor cells. In order to produce energy normal cells use mitochondrial oxidative phosphorylation[162, 163]. In contrast, cancer cells have an increased glucose uptake and an enhanced glycolysis. In the recent years it has become evident that reprogramming of cancer metabolism is more complex than believed and the so-called Warburg effect has been challenged. Importantly, altered metabolism in cancer cells may not be a consequence but play a more active part in tumorigenesis [164].

MYC has also been reported to have major effects on metabolism by indirectly or directly targeting many genes involved in metabolism such as *lactate dehydrogenase A (LDHA)* and *ornithine decarboxylase (ODC)* [148, 160, 165]. It has been shown to directly or indirectly regulate genes involved in aerobic glycolysis such as glucose transporters. [148, 152]. Under non-hypoxic conditions MYC regulates glycolysis without HIF-1α, while it does co-regulate glycolysis with HIF-1 under hypoxic conditions [166]. Furthermore *MYC* overexpression has been reported to increase glutamine uptake by an enhanced number of glutamine transporters [149]. It has been demonstrated for tumors cells as well as for *MYC* overexpressing cells that these are dependent on glutamine in order to maintain the processes within the mitochondria [167, 168]. MYC has also been shown to positively regulate mitochondrial biogenesis [169-171]. Deletion of *MYC* leads to reduced oxidative phosphorylation, probably due to the reduced size and number of mitochondria. A link between MYC induced proliferation and increased mitochondrial function has been shown [172]. In addition, MYC has been reported to be involved in lipid metabolism. Our group has demonstrated in neuroblastoma cells accumulation of lipid droplets after the inhibition of MYCN or with inhibitors against component of the fatty acid oxidation [173].

1.5. DEREGULATION OF MYC AND ITS INVOLVEMENT IN TUMORIGENESIS

The *MYC* oncogene has been found to be deregulated in a large number of human cancers (Figure 6). Different mechanisms for deregulation of the *MYC* gene have been identified [14, 58, 77, 174]. *MYC*, which is located on chromosome 8, was found to be translocated to one of the *Immunoglobuline (Ig)* loci on chromosomes 14, 2 or 22 in BL. Mutations in the TAD of MYC especially in residues T58 and S 62 can only be found in BL [77]. Additionally, translocations of *MYC* have been reported for several forms of leukemia. Mutations in *MYC* are rare compared to other oncogenes such as HRAS. However, insertional mutagenesis has been reported for *MYC* and seems to be more abundant. If during the process of insertional mutagenesis, retroviral promoters or enhancers are inserted into the genome close to an oncogene this will lead to the overexpression of the oncogene [175]. The third possibility is amplification of one of the *MYC* genes, which can be found in cervical, colon cancer or osteosarcoma for *c-MYC* and in neuroblastoma and medulloblastoma for *MYCN* [175].

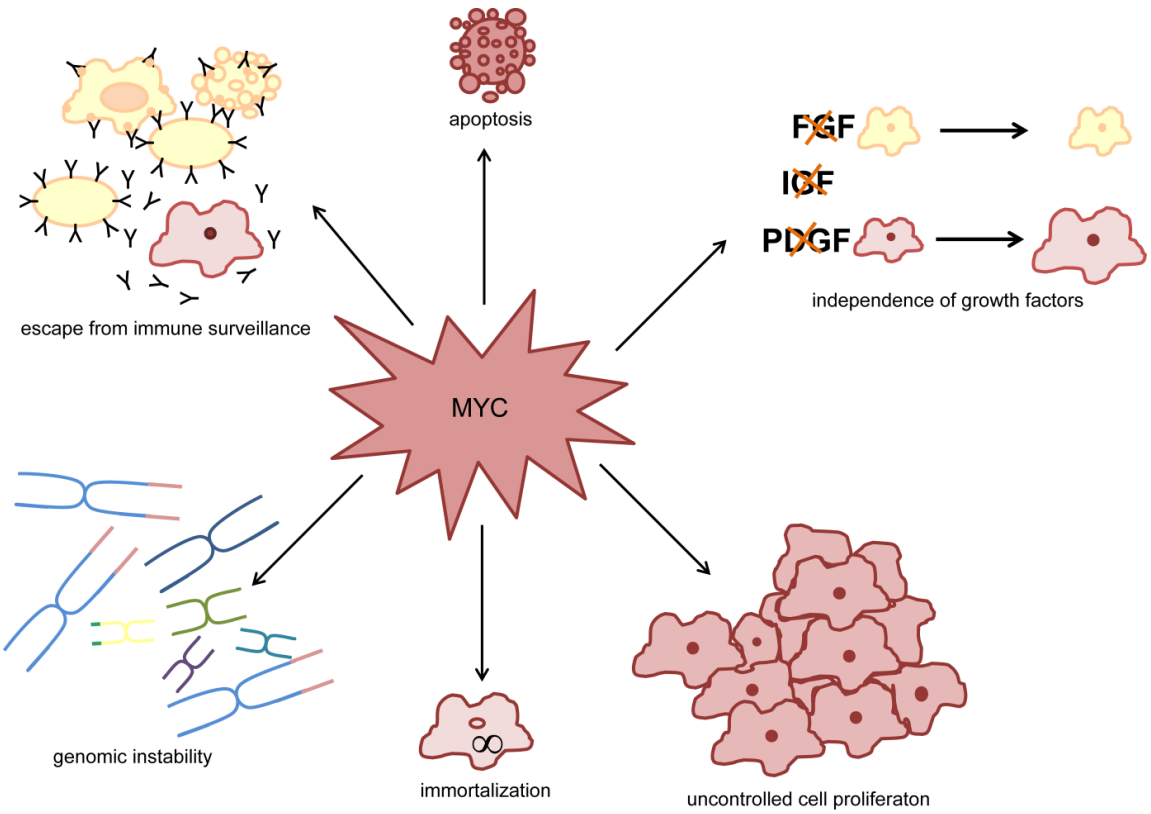

Figure 6. Outcome of MYC deregulation. *Aberrant MYC expression leads to uncontrolled proliferation, immortalization, escape from immune surveillance, independence from growth factors, genomic instability but can also result in apoptosis.*

The involvement of MYC in several cellular processes including apoptosis, cell cycle progression can explain the impact of its deregulated expression leading to e.g. a transformed cell with uncontrolled proliferation. In order for MYC to be successful in transforming cells, its

apoptotic function must be suppressed. This can either be achieved by keeping up the pro-survival and growth factor signaling or by inhibiting pro-apoptotic signals [24, 73, 125].

It has been shown that transformation of hematopoietic progenitor cells, breast epithelial cells and pancreatic β-cells can be induced by co-expression of *BCL-2* and *c-MYC* resulting in the suppression of apoptosis [73, 176]. Another mechanism to inhibit MYC-induced apoptosis by increasing MYC protein stability and its degradation [152]. RAS activates RAF/MEK/ERK pathway and by phosphorylation of S62 stabilizes MYC. Furthermore the PI_3K/AKT pathways is activated by RAS leading to the negative regulation of GSK-3β, which would phosphorylate T58 resulting in MYC ubiquitination and degradation [73]. Both, *RAS* and *MYC* are able to transform rat embryo fibroblasts. Furthermore they have been found to be altered in BL as well as promyelocytic leukemia. In several tumors, apoptosis induced by *MYC* overexpression can be suppressed by mutation of the *p53* gene leading to the interruption of apoptotic signaling [152, 177].

In order to study deregulated *MYC* in mice several transgenic mice models have been established. Since *MYC* overexpression leads to the death of the embryo, regulation of the *MYC* gene through insertion of an inducible or a repressible system can be achieved. Furthermore the results of *MYC* overexpression can be investigated in different type of tissues[178, 179]. Inactivation of *MYC* in the developed tumor first leads to tumor regression in all tumors. However the regression is dependent on the cell type and the genetic context. While in T-cell lymphoma the regression is due to cell cycle arrest, apoptosis and differentiation, the cells undergo differentiation in osteosarcoma [180]. Also the duration of *MYC* inactivation is dependent on the tumor type. Lymphoma cells first differentiate and then senesce after inactivation of *MYC* and finally undergo apoptosis [180-182]. In osteosarcoma the cells also first senesce or differentiate into bone cells and after reactivation of *MYC* apoptosis is induced in these cells [181]. Hepatocellular carcinoma cells undergo senescence, differentiation and apoptosis upon *MYC* deactivation [180]. However, its reactivation can lead to reappearance of the tumor [181]. Inactivation of *MYC* was also achieved by expressing a dominant negative mutant called *OmoMyc*, encoding for the bHLHZip domain of *c-MYC*. The OmoMyc protein dimerizes with the bHLHZip domain of wild-type c-MYC thereby inhibiting heterodimerization with MAX [183-185]. In *K-RAS* driven lung cancer this inactivation resulted in tumor regression with mild effects on normal proliferative tissue such as colon and intestine. These were encouraging results for MYC as a therapeutic target in cancer [183-185].

1.6. BURKITT'S LYMPHOMA

The first report describing Burkitt's lymphoma (BL) as a lymphosarcoma of young children in tropical Africa is from the year 1958 by Dennis Burkitt. Shortly it has been discovered to be a highly aggressive non-Hodgkin's Lymphoma. Three different subtypes of BL have been

described including the endemic, sporadic and immunodeficiency-associated variant [186, 187]. All three subtypes have the chromosomal rearrangement of the *c-MYC* gene to one of the *immuneglobuline* (Ig) loci in common leading to the overexpression of *c-MYC*. However, there are clear differences between these three variants [188].

Endemic BL occurs in children in the holoendemic malaria areas of Africa and the mean age of eBL peaks between 6-8 year old children. Tumors in children occur in the jaw, the eye and the abdomen [187, 189, 190]. The co-factors in endemic BL (eBL) are malaria and Epstein-Barr virus (EBV) infection. Malaria antigens are thought to protect B cells carrying translocations from apoptosis and/or to increase infection of those B cells with EBV [191, 192]. In the holoendemic malaria areas all children are repeatedly infected with malaria parasites and they also carry EBV. But it is still unclear why some children develop eBL, while the majority of children do not develop eBL [191, 192].

While the co-factor for the immunodeficiency-associated BL is the human immunodeficiency virus (HIV), no co-factors are known for the sporadic BL (sBL). Both sporadic and HIV-associated BL are occur worldwide and are not dependent on the age of the patient. In both forms less than 40% of the tumors contain EBV leading to the assumption that other co-factors are involved in the development of the disease. In sBL and HIV-associated BL tumors are mostly detected in the abdomen [186, 193].

Treatment of BL is normally short-duration combination chemotherapy together with aggressive central nervous system (CNS) prophylaxis. Previously it has been thought that children with BL had a better survival rate than adults. However it has been shown that chemotherapy together with CNS prophylaxis is as curative for adults than for children. However, the outcome of the treatment very much relies upon the stage of the disease [186, 193].

1.7. NEUROBLASTOMA

1.7.1. Clinical representation

Neuroblastoma (NB) is a rare cancer, but it is the most common extra cranial solid tumor in children. In the US it accounts for about 7-10% of all cancers in children and it is responsible for about 15% of pediatric oncology deaths [194, 195]. While there are around 700 new children estimated in the US to develop NB, 15-20 new cases are reported in Sweden per year [196, 197]. The diagnosis for most NB is made before the age of 5 years with a median occurrence of 22 months [198, 199]. The tumors arise from the developing neural crest and are most commonly found in the abdomen. The neural crest gives rise to peripheral neurons, enteric neurons, glia, melanocytes, Schwann cells and adrenal medulla (Figure 7). The tumors are associated with the adrenal gland or the sympathetic ganglia [200, 201]. NB is a very heterogeneous disease that can be divided in different groups including spontaneous regressing tumors, treatable differentiated

tumors but also rapidly growing, metastatic tumors. The last group is mostly resistant to treatment and associated with poor survival [200, 202]. Children that are diagnosed with tumors under the year of one and/or with localized tumors are normally curable with surgery and/or chemotherapy. In contrast, in children above one year, treatment of NB has proven to be more difficult. These children often have metastasis and outcome is mostly poor [203]. Some cases of familial neuroblastoma have been identified [204]. The *anaplastic lymphoma receptor tyrosine kinase* (*ALK*) has been found mutated in all cases of familial NB leading to its activation [205]. *Alk* is normally expressed during the development in a specific area of the brain in mice, while it can hardly be detected in the adult mice [206, 207]. While *MYCN* amplification (MNA) as well as loss of 1p or 11q are markers for poor prognosis, low MYCN together with high TrkA expression has been seen for tumors with favorable outcome [202, 208].

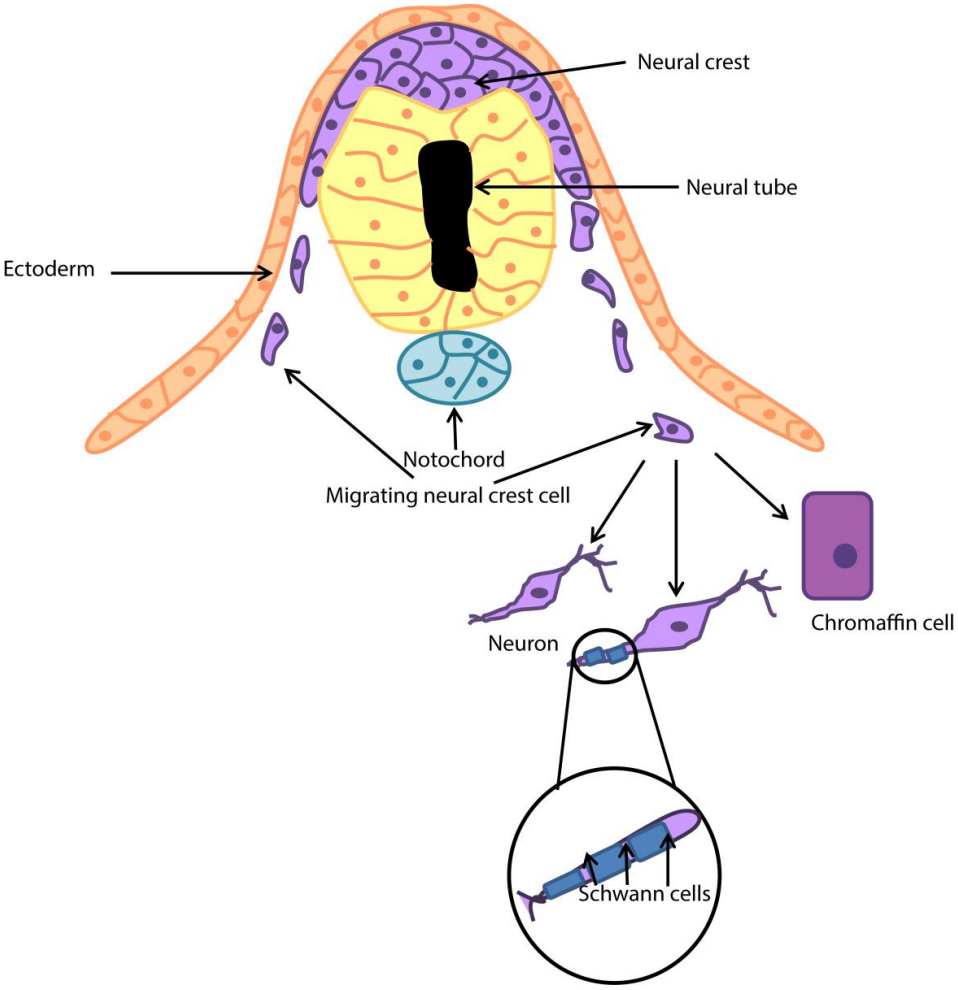

Figure 7. Development of cells derived from the neural crest. *The neural crest consists of multipotent stem cells, which undergo epithelial-mesenchymal transition (EMT) while they are migrating to the periphery of the embryo. During migration these cells are exposed to different signals resulting in a more differentiated cell. Neural crest cells can give rise to different kind of cells including swann cells, neurons and adrenal chromaffin cells. NB is believed to be derived from the sympatoadrenal lineage of neural crest cells.*

1.7.2. Genetic changes

In NB several genetic changes have been found including gene amplification, loss or gain of chromosomal loci or some few mutations. *MYCN* is amplified in about 25% of all primary NB's [70, 209]. Amplification of *MYCN* is a reliable marker for advanced stage, poor outcome and rapid tumor progression, whereas in non-*MYCN* amplified high MYC signaling could be correlated with high risk NB (132,136). It also has been found in combination with other alterations found in tumors with poor prognosis such as chromosome 1p deletion or gain of 17q as well as increased expression of multidrug resistance protein (MRP) [210-212]. Deletion of the 1p36 region was found in over 70% of all NBs, while the loss of 11q and 14q also occur frequently. The gain of 17q has also been detected in NB [200, 201]. The gain of whole chromosomes in NB is normally associated with a favorable outcome [194]. Gene amplification and somatic activating mutations of *ALK* have also been found in about 8% of NB, which in combination with *MYCN* amplification have been associated with adverse outcome [195, 213]. Elevated expression levels of *survivin*, *repp86* or *PRAME* have been correlated with poor outcome, while expression of the *neutrophin receptor TrkA*, *CD44* or *CAMTA1* has been reported for patients with a favorable diagnosis [214].

The prognosis in NB patients with young age (< 18 months) as well as a differentiated histology has been reported advantageous [215]. NB is normally treated in a combination of surgery, chemotherapy, radiotherapy and bone marrow transplantation depending on the risk group [216]. Also treatment with13-cis-retinoic acid is used in the clinic in order to induce differentiation. Here, down-regulation of MYCN could be observed prior to differentiation [70]. Despite recent progress in treatment the efficiency in treatment for high risk NB has improved little, resulting in a survival rate of approximately 15-30% [194, 215]. Even though NB's are initially responding to chemotherapy, drug resistance is frequently developed in high-risk patients [195, 203, 217, 218]. Therefore more tumor specific treatments and new strategies are needed in order to improve the survival and to decrease the side effects for the patients. Besides new compounds for e.g. down-regulation of MYCN or ALK, other targets such as miRNAs could be developed in order to treat NB. Two different approaches have been described in **paper I** and **paper III** [219, 220].

1.8. Targeting MYC in cancer

Targeting MYC has long been thought to be not possible due to its important roles in many different cellular processes necessary for normal cellular functions. Furthermore, no defined binding cleft can be found in the MYC protein, since it is intrinsically disordered. Successes in the development of molecules targeting protein-protein interactions (PPI) or enzymes have in most cases been due to a structured protein and probably also an available tertiary structure for compound modeling (106,107). However, recent results have supported the view that MYC can

be targeted in tumors without damaging normal cells and tissue irreversibly [183]. Since MYC-driven tumors are often associated with fast progression and poor outcome like in NB.

Different strategies to target MYC directly or indirectly have been developed [221, 222].The expression of *MYC* can be altered on both DNA and mRNA level by interfering with the *MYC* promoter or the transcription using e.g. siRNA or antisense nucleotides [14]. Another possibility is targeting of the MYC protein itself and/or its interaction partners with small molecules. By inhibition of the heterodimerization of MYC and MAX or binding of one of the binding partners to MYC, it will not be able to exert its functions properly [14, 221, 222]. A set of small molecules has been identified that interfere with the MYC/MAX interaction. These molecules bind to the bHLHZip domain thereby preventing MAX binding to MYC. Binding of the MYC/MAX complex to DNA could also be interfered with. Furthermore degradation of MYC protein could be increased. MYC target genes could also be inhibited either by modulating their expression or by inhibiting their protein products using siRNA or small molecules respectively [59, 223].

One example for an indirect way of targeting MYC is inhibition of BET bromodomain proteins, which are binding to acetylated histones in chromatin and increasing the number of transcription activators leading to transcriptional activation. Targeting this protein family by JQ1, a bromodomain inhibitor, has been reported to down-regulate MYC transcription and as a result also MYC target genes [224, 225].

A number of MYC regulated miRNAs have been identified during the last years. One miRNA induced by both c-MYC and MYCN is miR-9, which has been shown to play a role in metastasis and invasiveness of tumors [33]. Furthermore members of the miRNA cluster 17-92 have been reported to be involved in supporting induction of lymphoma in Eμ-*myc* mice by c-MYC. Additionally, we could show that MYCN induced the same cluster in neuroblastoma cell lines miR-18a and miR-19a suppressed *estrogen receptor alpha* (*ESR-1*) inhibiting differentiation of these cells (**paper III**) [33, 220, 226-228]. The delivery of RNA based miR inhibitors has been difficult. However, cholesterol linked 2' OMe oligonucleotides called antagomirs have been developed. Treatment with an antagomir for miR-10b could reduce the formation of metastasis in a breast cancer mouse model. Another method is to use locked nucleic acid-modified oligonucleotides so called LNA-anti-miRs and they have been reported to down-regulate their respective miR target in mice [30, 229].

Despite the difficulties of targeting IDPs, several small molecules interfering with the c-MYC/MAX interaction have been identified [221, 230]. Most of these compounds have been found in a two-hybrid yeast screening by Edward Prochowniks' group. Treatment of c-MYC overexpressing cells with a selection of these compounds resulted in cell cycle arrest, inhibition of cell growth and promoting apoptosis [231, 232]. Binding to the bHLHZip of c-MYC has been confirmed using a number of biophysical experiments [233-235]. However, there have also been some issues including solubility and fast turnover and probably also as a result these

compounds were found to not be effective in xenograft model for prostate cancer [236, 237]. Interestingly, our group demonstrated *in vivo* effects of the most investigated compound 10058-F4 in a MYCN transgenic tumor model of neuroblastoma [173]. We could further show that inhibition of MYCN resulted in lipid droplet accumulation in NB cells. Our group was confirmed the binding of all small molecules previously shown to bind to c-MYC to MYCN, probably interfering with the MYCN/MAX heterodimerization leading to lipid accumulation, differentiation and inhibition of cell growth [173, 219].

An indirect way of targeting MYC can be achieved by inhibition of mitotic processes like Aurora kinase inhibitors, which has been shown to induce lethal therapeutic responses in mice. The most promising molecule so far is JQ1, an inhibitor of Bromodomain containing 4 (BRD4) proteins, which have been shown to be involved in MYC induced transcription. JQ1 has been shown to prolong survival in several NB mice models [238].

2. MAIN EXPERIMENTAL BIOPHYSICAL METHODS

2.1 *C*ircular *D*ichroism (CD)

Using the spectroscopic technique called Circular dichroism (CD) differences in the absorption of left and right circularly polarized light through chiral compounds are measured. The proportion of polarized light is never the same. CD is normally used to determine secondary structure of proteins or peptides. It can also be used to determine secondary structure changes upon interaction of two binding partners. Secondary structure measurements are normally performed in the far UV range (240-180 nm) [239]. In this UV range the peptide contribution to the CD spectra dominates. Different structural elements have a unique spectrum with minima or maxima at specific wavelength. The spectra of an antiparallel β-sheet has a minimum at 218 nm and a maximum at 195 whereas the spectra of a completely α-helical protein two minima at 222 nm and 208 nm with one maximum at 193 nm. Disordered, extended proteins have little ellipticity above the wavelength of 210 nm. The CD spectra of proteins can be used to determine their secondary structure content as well as to examine their structural changes due to temperature changes, denaturating solutions or mutants. However, CD is not able to determine the exact residues for the secondary structure identified and is not a high resolution method such as NMR or X-ray crystallography [239-241].

2.2 *S*urface *P*lasmon *R*esonance (SPR)

Surface plasmon resonance (SPR) is a technique based on an optical method using the refractive index close to the sensor surface for the detection of binding between two molecules. The most known instruments are the BIAcore machines [242]. The surface of a sensor chip in the machine will create small flow cells (Figure 8A). All sensor chips are coated with a very thin gold layer, where one of the binding partners the so called ligand is immobilized to. There are different chips for different methods to immobilize one of the ligand to the surface of the chip using amine-, thiol- or aldehyde coupling. There are even chip for capturing His-tagged proteins [243]. During all steps a buffer is continuously flown over the surface with the same speed . In order to determine binding the analyte is injected in increasing concentrations leading to a concentration dependent increase in the refractive index when the analyte is binding to the ligand. The change in the refractive index can be monitored and is visualized by a sensorgram (Figure 8B). The sensorgrams can be fitted through mathematical formulas enabling the determination of the rate of association and dissociation as well as the affinity. The system allows the analysis of proteins, low molecular weight compounds, lipids, nucleic acids, carbohydrates as well as cells, viruses and bacteria [242-244].

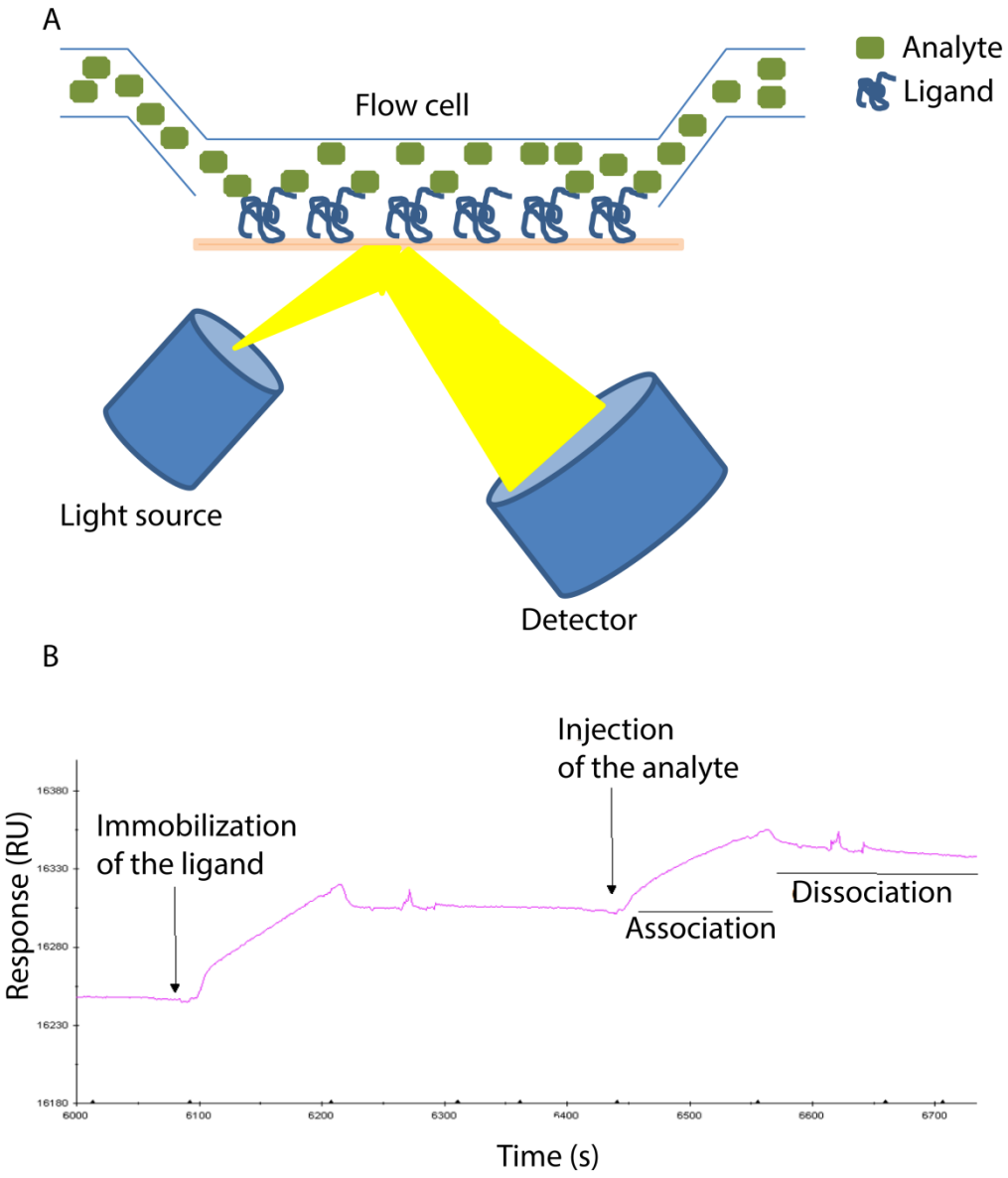

Figure 8. Concept of Surface Plasmon Resonance. A) *The ligand is immobilized on the surface of the sensor chip. The analyte is flown over the surface. A light beam is directed towards the chip, leading to a change in the refractive index after the analyte has bound to the ligand. This response is visualized in a sensorgram.* ***B)*** *The ligand (here an His-tagged protein) is immobilized on a NTA-chip. After non-bound ligand is washed and the signal is steady, the analyte is injected. Association and disassociation of the analyte to its ligand can be seen.*

2.3 Small angle X-ray scattering (SAXS)

Small angle X-ray scattering is a low-resolution method for the structural characterization of materials such as detergents, synthetic- and bio-polymers, organic or inoraganic film and biological macromolecules in solution. A monochromatic and collimated X-ray beam is directed onto the sample solution (Figure 9). The scattering pattern is recorded by a X-ray detector.

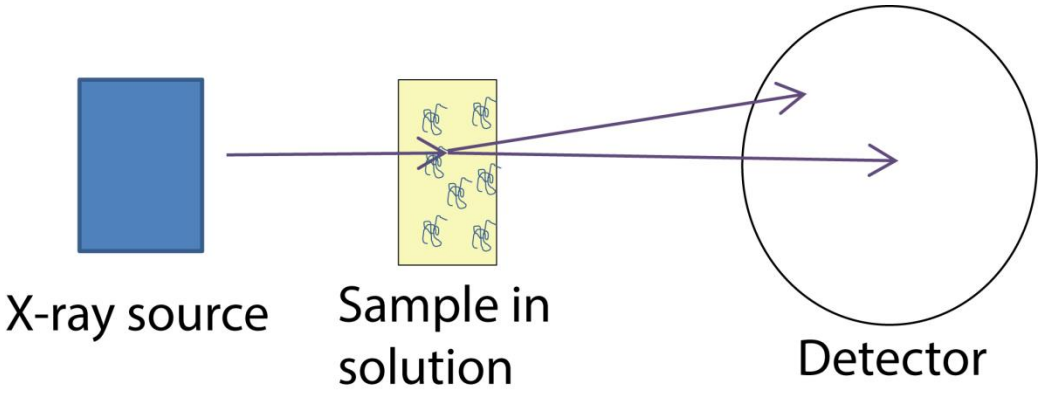

Figure 9. Scheme of a small-angle X-ray scattering experiment. *A monochromatic X-ray beam is directed to the protein in solution and scattered. The scattered beams are collected by a detector.*

The macromolecules investigated can be quite large up to some Giga Dalton. SAXS allows for a quantitative analysis of equilibrium mixtures. In addition, SAXS provides insights into the overall size of the protein or complex in solution, their shape, the structural arrangement of domains in a protein as well as an estimation of the flexibility of a protein and the different conformations, it can adopt [245]. There are several advantages with using SAXS for gaining structural insights. Besides being able to gain information about size and shape of proteins in solution, structural information can also be used for intrinsically disordered proteins or the fold of denatured proteins. Furthermore intermediate structures of proteins could be determined. A disadvantage is the low resolution that does not provide as in-depth structural details as X-ray crystallization [245, 246].

2.4 X-ray crystallization

The determination of the protein structure down to its atoms can be investigated by X-ray crystallization. For this purpose high protein purity and high concentrations are needed. The conditions, under which the proteins crystallize cannot be predicted. The bottle-neck in X-ray structure determination is the crystallization of the protein. Several aspects of the buffer are important for proteins to crystallize including ionic strength, the type of the ion (anions, cations), the pH of the solution, polymers such as polyethylene glycol in solution, organic acids, additives and the concentration of both protein and the components of the buffer [247]. In addition, the temperature is an important factor. For a protein to form a crystal, the right equilibrium between protein concentration and the concentration of the precipitant solution must be determined. Protein crystals grow by slow controlled precipitation of the protein in aqueous solution in conditions that do not denature the protein (Figure 10). While the crystal is formed, intramolecular hydrogen and ionic bonds form and water is released, since some hydrogen bonds are sacrificed. On the surface of the protein in solution hydrophobic patches are lacking favorable hydrogen bonds. However, they are surrounded by ordered unfavorable water

molecules. Therefore, the hydrophobic patches are normally buried by intermolecular interactions such as dipole-dipole or Van-der-Waals forces [248].

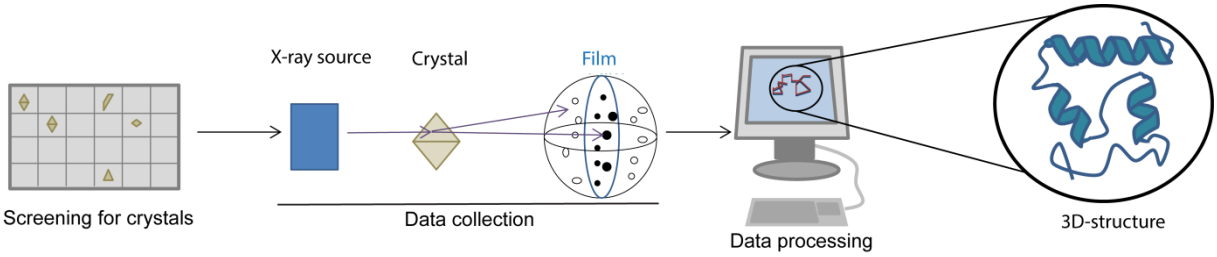

Figure 10. The process from crystal screen to a 3D-structure. *The screening for crystallization conditions leading to the crystallization of the protein is followed by data collection. A monochromatic X-ray beam is diffracted by the crystal. These reflections (black circles), which can vary in their intensities are collected by a detector and visualized on a film. Not all reflections (white circles) can be detected due to the position of the detector. Therefore data will be collected at different angles. Afterwards the data obtained is processed using several programs for structural determination.*

The crystal is mounted between a X-ray source and a X-ray detector, on which the diffracted beams can be seen as pattern. The diffractions are the sums of the electrons from the atoms in a protein. The diffraction patterns of different angles of the crystal ranging from at least 30-180 degrees will be obtained in order to be able to construct a 3D-structure. The symmetry of the crystals determines the amount of data that is required to solve a structure (Figure 10).

The visualization of the structure of the protein the phase problem must be solved. This can be achieved using different methods such as Molecular Replacement, Multiple Isomorphous Replacement (MIR) or Multiwavelength Anomalous Dispersion (MAD). The results are electron density maps, which can be used for the building of the model. However, very flexible regions might not be visible. All the data is processed using different programs for visualization. The preliminary model will be refined against the data, which will lead to a better model. This step might be repeated several times until no further improvements can be seen and a model of the protein structure can be obtained [249-251].

ETHICAL STATEMENT

This thesis contains work with neuroblastoma and fetal tissues. These ethical permits are specified in paper III.

3. AIMS OF THE STUDY

The aims of this thesis was to characterize the structure of the c-MYC transactivation domain (TAD) using biophysical and biochemical methods as well as to investigate several small molecules in order for direct targeting MYCN in human cancer cells.

The thesis is based on four different papers. In **paper I** several small molecules known to target c-MYC were characterized for their binding ability to MYCN as well as their effects on apoptosis, differentiation and lipid accumulation in *MYCN*-amplified neuroblastoma (MNA-NB) cells. Furthermore, screening, identification and characterization of a set of several small molecules directly or indirectly targeting c-MYC and/or MYCN was performed in **paper II**. These molecules were tested for their ability to interfere with MYC/MAX interaction. Furthermore, the effect of these compounds was analyzed for effects on proliferation, anchorage-independent growth and foci formation. The aim of **paper III** was to identify and characterize MYCN regulated miRNAs in neuroblastoma and to analyze the functional outcome of the interaction. Importantly, the focus of **paper IV** was to investigate the structure of the c-MYC TAD using biophysical methods including Circular Dichroism, Small-angle light scattering and X-ray crystallography.

4. RESULTS AND DISCUSSION

4.1. Paper I. Targeting of the MYCN protein with small molecule c-MYC inhibitors

Targeting protein-protein interactions has been a more challenging task than finding a suitable compound inhibiting enzymes. For all protein-protein interactions (PPI), several domains of the interaction partners can be involved in the binding, which can be difficult to target. Progress has been made, however the traditional approach for drug development targeting ordered proteins has been through their three-dimensional structure. This resulted in the assumption that IDP's should be excluded as potential drugable proteins. However, recently it has been shown that the interaction between disordered and ordered (e.g. the p53/Mdm2interaction) or interactions that undergo a disorder-to-order transition (e.g. the c-MYC/MAX interaction) can indeed be targeted. The aim of this study was to investigate the ability of known c-MYC inhibitors for binding to MYCN using SPR. Furthermore, the impact on known MYC functions after treatment with small molecules on MNA-NB cells was investigated in order to estimate their potential in cancer therapy.

Several small molecules targeting the c-MYC/MAX interaction have been described [252, 253]. Especially, compounds discovered in Edward Prochownik's group have been the basis for biophysical studies demonstrating direct binding to the bHLHZip domain of c-MYC [233-235]. Among these 10058-F4 is the most studied molecule showing inhibition of c-MYC/MAX binding as well as the ability to cause cell cycle arrest, apoptosis and inhibition of cell growth in a c-MYC dependent manner in tumour cell lines [231, 232]. However, 10058-F4 had not been able to inhibit tumor growth in a prostate xenograft model overexpressing c-MYC [237]. In contrast our group demonstrated a significant anti-tumorigenic effect could be demonstrated in a xenograft model using *MYCN*-amplified (MNA) cells. Furthermore, treatment of MNA-amplified NB cell lines with 100058-F4 resulted in induction of apoptosis, neuronal differentiation and the accumulation of lipids [173]. Impairment with the mitochondrial metabolism by targeting MYCN leading to lipid accumulation might be a new strategy for future treatment of high-risk NB.

Encouraged by these previous findings for 10058-F4 in NB cells, we next wanted to analyze the direct binding of selected molecules to the bHLHZip domain of MYCN. To this end, we chose 10058-F4, three of its analogs, the 10058-F4 metabolite C-*m/z*232 as well as the structural unrelated compound 10074-G5, which binds to a different site in the c-MYC bHLHZip domain than 10058-F4. These molecules were investigated for their affinity to MYCN using SPR and their ability to interfere with the MYCN/MAX heterodimer was investigated with a proximity ligation assay (PLA). Furthermore functional effects of treatment with these compounds on growth inhibition, apoptosis, neurite outgrowth and lipid accumulation was determined.

The C-terminal bHLHZip domain of the MYC family proteins is disordered but folds upon binding to MAX. In addition, the molecules investigated have a low solubility and needed to be

dissolved in DMSO increasing the difficulty to find a method suitable to detect protein-molecule binding, since DMSO interferes with some biophysical methods. Therefore SPR was chosen in order to analyse binding. Affinities to c-MYC as well as MYCN could be determined for all molecules, except for the analogs 10058-F4(7RH) and #764. While 10058-F4(7RH) was previously reported not to bind to c-MYC and therefore was used as a negative control, the affinity for #764 could not be determined. This might be due to the low solubility of the compound and the temperature settings of the assay at 25°C. However, a concentration dependent increase in the curves of the sensorgrams could be detected indicating binding of #764 to the bHLHZip of MYCN and of c-MYC, respectively. The affinities to MYCN were the highest for 10074-G5, followed by #474, 10058-F4 and C-*m/z* 232. The standard errors were quite large for the SPR assay, which may have several reasons. The evaporation of the samples during the preparation process and the solubility of the compounds as well as the possibility that they might be precipitating during the assay run could have an effect on the affinities of the molecules to MYC. Furthermore, the Biacore machine used for the assay is very sensitive to very small differences in the concentration of compounds within the buffer or the compound solution. Despite high standard error values, the affinities obtained by SPR were higher than those previously described in the literature [233, 235, 254]. One possibility might be the different methods used to determine the affinities. However there have also been studies indicating that the affinities might be lower than those previously reported. Importantly, a correlation between the affinities of the small molecules to the IC_{50} values determined for inhibition of cell growth could be observed. This correlation indicates that the strength of the interaction between the molecules is important for the efficiency to inhibit cellular growth.

To further evaluate the ability of the molecules to interfere with the MYCN/MAX heterodimer, a PLA assay was performed in MNA-NB cells after 6 hours of compound treatment. In this assay the proximity of two proteins is detected by the use of two specific antibodies directed against each of the proteins. Specially designed secondary antibodies with a short unique DNA strand attached are used for visualization of protein-protein interactions. When two proteins interact the DNA strands come into close proximity and form a circle, which is being amplified. A fluorescent detection probe is attached to the DNA and the signal is detected by a fluorescence microscope. The assay revealed that the compounds 10058-F4, 10074-G5, #474 and #764 were able to decrease the interaction between MYCN and MAX. In contrast, C-*m/z* 232 did not have any effect on the MYCN/MAX heterodimerization. This might be due to a low affinity in order to prevent binding of MAX to MYCN. Importantly, the non-binder 10058-F4(7RH) did not show any effect on the heterodimerization of MYCN and MAX. The same molecules decreased the interactions of MYCN and MAX, were also the most potent in inducing apoptosis in a doxycycline regulated MYCN-expressing cell line Tet21N. After treatment with molecules increased apoptosis in MYCN-expressing cells compared to cells with low MYCN levels could be observed. Again the 10058-F4 metabolite was not able to induce apoptosis in MYCN-expressing or in non-expressing cells indicating that the compound 10058-

F4 is responsible for the effects observed *in vivo*. Both analogs (#474 and #764) were the most effective in inducing cell death.

In order to investigate the effects of the molecules on MYCN protein levels Western blot analysis was performed. Only 10058-F4 and 10074-G5 were found to reduce MYCN protein levels. The small molecules #474 and #764 were most effective compounds in inducing apoptosis, while treatment with 10058-F4 and 10074-G5 resulted in neurite outgrowth and lipid accumulation in MNA NB cells.

The two molecules 10058-F4 and 10074-G5 were able to reduce MYCN/MAX interaction and to decrease MYCN protein levels. Furthermore, treatment with either of these two compounds lead to enhanced apoptosis in MYCN-expressing NB cells, neurite outgrowth and lipid accumulation. The two analogs #474 and #764 were able to interfere with MYCN/MAX binding. They were also very potent in inducing apoptosis at low doses however no effect on protein levels could be shown. After treatment with one of the analogs neither neurite outgrowth nor lipid accumulation could be observed in MNA NB cells. This might indicate that these processes occur as a result of MYCN down-regulation in MNA NB cells. The metabolite *C-m/z 232* showed the lowest affinity and was not able to interfere with the MYCN/MAX heterodimerization or to induce apoptosis. Compared to 10058-F4 and 10074-G5 the effect on neurite outgrowth was less pronounced after treatment with high concentrations. However the metabolite was able to induce some lipid accumulation but only after treatment with higher concentrations indicating that this might not be due to a specific response to MYCN inhibition but rather due to stress induced by the high concentrations of compound present in the cell.

In summary, we showed that the small molecules previously shown to bind to c-MYC also bound directly to MYCN. Interference with the MYCN/MAX heterodimerization was induced MYCN-dependent apoptosis in MNA NB cells. Furthermore, the decrease of MYCN/MAX interaction also leads to other MYCN-selective responses such as lipid accumulation and differentiation following the decrease in MYCN. MYC-driven tumors are often associated with poor prognosis and survival. In the latest years the possibility of targeting MYC directly in cancer has become evident[183]. Several groups are currently screening for small molecules that directly target MYC. Furthermore already existing compounds are being improved regarding their solubility, their cellular uptake and their affinity to MYC [237, 254, 255]. Here we have shown as proof of principle that targeting MYCN in NB is possible and might be a new treatment strategy for high risk NB.

4.2. Paper II.Identification of low molecular weight compounds that target the MYC:MAX protein interaction and inhibit MYC-dependent tumour cell growth

The compounds described in **paper I** have been so far the most promising small molecules targeting MYC. To date there are no drugs present targeting MYC in the clinic since the compounds available until now show low potency at high concentrations and/or off-target effects. Therefore more potent MYC targeting molecules with less off-target effects are needed. In this study a cell based screening assay was performed identifying several potential small molecules interfering with the MYC/MAX interaction[221].

First the cell-based Bimolecular Fluorescence Complementation assay (BiFC) was chosen as a method for an initial screening of a chemical library for interference with the MYC/MAX heterodimerization. For the screen the yellow fluorescent protein is fused into two halves. While one half is fused to c-MYC the other is attached to MAX. When these two proteins come into close proximity the YFP halves can fuse and start to fluoresce, which can be detected using a fluorescence microscope. After optimization, 2000 small molecules were screened and 15 compounds were identified in the first round.

These hits were then validated in a Gaussia luciferase protein fragment complementation assay in HEK293 cells using the same principle as in the BiFC assay. In this refined screen, the ability of the compounds to disrupt both c-MYC/MAX as well as MYCN/MAX heterodimerization was assessed. Out of 15 compounds six (#2, 6, 7, 9, 11 and 14) were found to decrease MYC/MAX and MYCN/MAX interaction.

All six compounds were then tested for their ability to directly target the MYC/MAX and MYCN/MAX interactions by mixing the fusion proteins and measure the effect of the small molecules on heterodimerization. While compound #2 was very efficient in disrupting MYC/MAX and MYCN/MAX *in vitro* it was less effective in cells, probably due to poor uptake, degradation within the cell or an efficient efflux. In contrast, the small molecule #7 showed little effects in this assay leading to the assumption that it might indirectly target the MYC/MAX interaction. Compounds #6, 9 and 11 were also able to inhibit MYC/MAX heterodimerization. Using Western blot analysis of treated cells the ability of the compounds to down-regulate protein levels was tested. Only compounds #7 and 14 could be shown to down-regulate MYC protein levels. It was further demonstrated that the compounds #2, 6, 9, 11 and 14 were not able to interfere with the interaction of neither FOS/JUN nor GCN4/GCN4 suggesting specific targeting for MYC/MAX.

All small molecules were shown to have inhibitory effects on cell growth in MNA NB cell lines using a WST-1 assay. Compounds #7 and 9 were more potent in growth inhibition than #2, 6, 11 and 14. While compounds #6, 7 and 11 were found to be more effective in MNA cell lines compared to non-*MYCN* amplified cell lines, no such effect could be observed for compounds

#2, 9 and 14 indicating that these molecules could interfere with MYCN as a transcription factor. Indeed, a reduction of β-gal activity greater than 50% was obtained for all compounds except of #9.

Growth inhibition could also be seen with compounds #2, 6, 7, 9, 11 and 14 in two BL cell lines. Two molecules, #6 and 7, were the most effective in both cell lines, while #2, 11 and 14 inhibited growth to a lesser degree. Compound #9 was effective in one of the cell lines.

Compound #2 was chosen for further optimization since it was shown to interfere directly with the MYC/MAX heterodimerization. This molecule was found to be less active in cells than in a direct protein-protein inhibition assay. Therefore analogs were selected from the NCI/NIH library and examined for their ability to interfere with the MYC/MAX and MYCN/MAX interaction in the GLuc assay. Inhibition was increased for analog #2:7 compared to its mother compound.

The effect of the analog on MYCN/MAX heterodimerization was further demonstrated using an *in situ* Proximity Ligation Assay (isPLA) with antibodies against MYCN and MAX. Again a fluorescent signal will only be seen if MYCN and MAX are in close proximity. Two MNA cell lines and one non-*MYCN* amplified cell line were investigated for MYCN/MAX heterodimerization with and without #2:7 treatment. As expected, the interaction of MYCN and MAX could only be detected in MNA-cells and the signal was reduced after treatment with #2:7.

Direct binding to the bHLHZip of both c-MYC and MYCN could also be shown for two analogs (#2:7 and 2:16) in an SPR assay, while #2 showed unspecific binding. In addition, treatment with compound #2:7 decreased transcription in a Luciferase assay using an E-box containing promoter and resulted in a reduction of proliferation. In addition, both foci formation and anchorage-independent growth were inhibited by #2:7.

In summary, six compounds were identified due to their inhibition MYC-dependent cell growth out of the initial 2000 molecules screened. The analog (#2:7) of the #2 mother compound was further characterized showing direct binding to the bHLHZip domain of both c-MYC and MYCN. In addition, this molecule was demonstrated to interfere with the MYCN/MAX interaction. Importantly treatment of NB cells resulted in a decrease in proliferation, foci formation and anchorage-independent growth. Here, a robust screening method has been used in order to identify new interesting molecules to target MYC directly or indirectly. The effect of these molecules could be further investigated in e.g. xenograft models in mice in order to investigate their efficiency *in vivo*.

4.3. Paper III. MYCN-regulated microRNAs repress estrogen receptor-α (ESR1) expression and neuronal differentiation in human neuroblastoma

One of the markers for poor prognosis and fast progression of NB is *MYCN*-amplification[256, 257]. However, the mechanisms underlying tumor development in *MYCN*-overexpressing NB is not well understood. In the recent years it has become clear that deregulated expression of small non-coding RNA's, called miRNAs is involved in tumorigenesis [32, 205]. Therefore, we investigated regulation of miRNA by MYCN in NB cell lines.

The Tet21N NB cell line with inducible MYCN-expression was used in a miRCURY Locked Nucleic Acid (LNA) array in order to identify MYCN-regulated miRNAs. Comparing MYCN-expressing to non-expressing cell lines we identified several down-regulated miRNAs in cells expressing *MYCN*. Additionally we found several up-regulated miRNAs including the *miR-17~92* cluster (miR-17, miR-18a and miR-19a). These results were confirmed by Northern blots using RNA from Tet21N cells as well as quantitative polymerase chain reaction (qPCR) analysis of human NB tumor samples. Direct binding of MYCN/MAX to upstream E-box sequences of the miR-17~92 cluster was verified by chromatin immunoprecipitation (ChIP). In addition, increased acetylation of histone H4 could be shown for these regions indicating that they are actively transcribed.

It had previously been shown that the *miR-17~92* cluster contributed to NB tumorigenesis and the involvement of two of its miRNAs, miR-17 and miR-20a had been examined [32]. However, the biological outcome of miR-18a and miR-19a overexpression had not been investigated. In order to test the impact of LNA knockdown of miR-18a and miR-19a in *MYCN*-amplified cells fluorescence activated cell sorting (FACS) analysis was performed. Knockdown of miR-18a lead to a robust decrease in cell cycle progression, while the effect was less pronounced after miR-19 knockdown. It has previously been shown that *MYCN* expression is down-regulated in differentiating neuronal cells and conversely that down-regulation of MYCN results in neuronal differentiation of NB in vitro [70]. Importantly, long-term knockdown of miR-18a also resulted in differentiation of MNA-NB cells. Downstream target genes of miR-18a and miR-19a were predicted using TargetScanHuman and PicTar. Interestingly one of the genes that scored high was the *estrogen receptor alpha (ESR1)*, encoding for the nuclear hormone receptor (ERα). The repression of *ESR1* expression by miR-18a and miR-19a was shown in a luciferase reporter assay in HEK293 cells which were transfected with *ESR1* 3´UTR sequence.

Transfection of the breast carcinoma cell line MCF-7 expressing high levels of *ESR1* with miR-18a or miR-19a precursor molecules lead to down-regulation of *ESR1* mRNA and estrogen receptor alpha (ERα) protein. In Tet21N cells ERα levels recovered after long-term repression of *MYCN* expression. Conversely, inhibition of MYCN protein levels was followed by reduction in miR-18a and miR-19a levels. The same effect could be demonstrated in MYCN3 cells, containing a *MYCN*-inducible expression system. In order to investigate the role of *ESR1* in NB cell lines, SK-N-BE(2) cells were transduced with *ESR1* cDNA resulting in growth arrest and neurite outgrowth. The expression of ERα in the sympathetic nervous system could be

demonstrated by immunohistochemistry staining suggesting a role during normal neuronal development. In addition we demonstrated a reverse relationship between expression of *MYCN* and *ESR1*. In patients, high expression of *MYCN* leading to low expression of *ESR1* is coupled to poor prognosis while low *MYCN* and high *ESR1* expression correlated with improved event-free survival using bioinformatical analysis of microarray data of NB tumors.

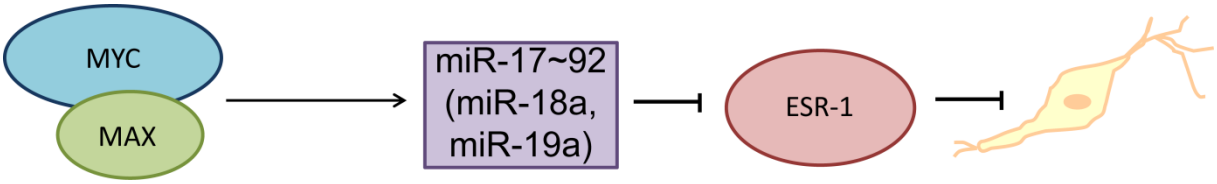

Figure 11. *Proposed mechanism of MYCN-regulated miR-17~92 cluster (especially miR18a and miR-19a), which in turn repress ESR-1 expression and as a consequence block differentiation in NB cells.*

Taken together, we showed that *MYCN* expression in NB cells leads to induction of the miR17-92 cluster and that miR-18a and miR-19a expression decrease of *ESR1* on mRNA and protein levels thereby inhibiting NB differentiation (Figure 11). Hence, targeting MYC-regulated miRNAs could be a new strategy for cancer therapy In MYC-driven cancers. High-risk tumors are often poorly differentiated and down-regulation of miR-18a may induce differentiation in *MYCN*-amplified NB. However, a suitable delivery system for anti-miRs often has to be developed before being able to use it in therapy.

4.4. Paper IV. Structural analysis of the c-MYC transactivation domain reveals highly flexible regions in an extended rod like structure

Intrinsically disordered proteins have been reported to be involved in human diseases due to protein misfolding resulting in loss of function, gain of a toxic function or protein aggregation. Targeting IDPs is challenging and little is known about their structures, since some only fold upon binding and others remain unstructured making structural determination difficult. Furthermore, IDPs are thought to be able to adapt different types of structures depending on their binding partners, leading to a variety of conformations that the protein might adapt. MYC is one of these proteins [45, 47, 49]. The C-terminal part of MYC has been shown to fold upon binding to its interaction partner MAX and the crystal structure is solved in complex with DNA [80]. In contrast, little is known about the structure of N-terminal domain, which has been reported to bind to a large number of binding partners [75, 78]. Previous structural and biophysical studies have determined secondary structure for smaller domains within the c-MYC-TAD. The c-MYC-TAD (residues 1-88) was reported to contain two short α-helical stretches as well as a short β-sheet stretch.

Here, we have used CD to estimate the secondary structure content of the c-MYC-TAD. Importantly, we investigated the structure of the c-MYC-TAD using SAXS and X-ray crystallization. The analysis of the c-MYC-TAD CD spectra obtained indicated 9 % α-helices, 30 % β-sheets and 61% unstructured domains. This is in contrast to the predictions based on the amino acid content revealed a higher content in α-helical structure (18%), much less β-sheets (3%) and 79% unstructured domains. However, the differences could be due to the fact that structural predictions are based on previously determined structures with homology. Since IDP's can take several different structures and might fold upon binding to their partners, it is difficult to estimate their secondary structure content in an unbound state. Therefore the prediction program might only predict one of many possible secondary structures. Furthermore in the unbound state the c-MYC-TAD might take several confirmations with varying secondary structure content due to the flexibility of the protein.

In order to analyze the overall structure of the c-MYC-TAD SAXS was used. Forward scattering revealed the presence of a pentameric complex in solution. In support the size exclusion profile showed a complex of 3-4 times the monomeric weight, after comparison with a kit containing different globular proteins with known molecular weight (MW). The discrepancy in oligomer size might be due to the difference in the methods. However, the c-MYC-TAD is a very flexible domain, which runs in a different manner through the same column compared to a more globular protein. The oligomeric state of the c-MYC-TAD might by itself lead to an increase in its structural content, which might account for the discrepancy between the predicted and obtained secondary structure. Importantly, we showed that the pentameric complex contained structured domains, which were connected by flexible linkers. The pair distribution constant revealed a single peak with a shoulder, indicating the presence of an extended and flexible protein. The overall shape fitted best to an elliptical cylinder.

In another approach we attempted co-crystallization experiments with a Fab-fragment, derived from the 6A10 monoclonal antibody in order to stabilize the c-MYC-TAD. First, binding after digestion of the antibody was determined using surface plasmon resonance (Biacore), revealing a high binding affinity in the low nM range. After microcrystals appeared in the fine screen, Western Blot analysis of one of the crystals indicated that both c-MYC-TAD and the Fab fragment might be present in the crystal. However, since the crystal dissolved immediately in solution, possible precipitations attached to the crystal could not be washed off, which may have lead to a false positive result. Two data sets from two different crystals were obtained, however after determination of the unit cell, the data could not be processed any further.

The Fab fragment is not a natural binding partner but a solved crystal structure of the c-MYC-Fab complex might still give insights on a possible confirmation as well as the possibility for the c-MYC-TAD of gaining secondary structure after binding to a stabilizing partner. Interactions between the c-MYC-TAD and its binding partners might be too weak for crystallization of larger regions. The possibility to determine a structure of such a complex might also depend on the secondary structure of the binding partner. However, methods such as SAXS paired with NMR and CD might also lead to more detailed structural information of the c-MYC-TAD alone as well as in complex.

In summary, we determined that c-MYC-TAD forms a pentameric complex in solution. Our analyses revealed an extended, flexible protein with structured domains connected by flexible linkers leading to an elliptic overall structure. It has previously been shown that the bHLHZip of c-MYC produced in bacteria can form tetramers. Here, we have shown that the c-MYC-TAD forms a pentameric complex in solution. This might indicate that the MYC protein indeed is able to oligomerize. However, this would have to be shown for the full-length protein and might lead to further insights of MYC function.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

During the recent years overall survival has increased in NB patients. However, not much progress has been made regarding the treatment of high risk NB (including patients with *MYCN*-amplification or 11q-deletion) and the survival rate for these children is still under 50%. Furthermore NB is responsible for 12% of all cancer related deaths in children [211]. Correlations between MYCN expression and the multidrug resistance-associated protein (MRP) have been described. Indications of activation of MRP by MYCN overexpression have been made, which could lead to acquired drug resistance and failed NB treatment [202, 212].

Most traditional strategies for small molecule development have been to target structured proteins such as the active domain of enzymes. In contrast, targeting IDPs e.g. MYC has been a challenging task since the flexibility of these proteins and their low structural content has been regarded as undrugable. Furthermore, the existence of a three-dimensional structure has of great help in drug design in the past and will be an important advantage also in future[47, 49]. Inhibition of MYC function *in vivo* using the dominant negative protein Omomyc protein has shown anti-tumorigenic effects in mice with reversible effects on normal, fast proliferating tissues. Therefore targeting MYC has become a desirable new approach for cancer therapy [183]. However, to date no drugs directly targeting MYC/MAX or MYCN/MAX can be found in the clinic due to their low solubility and/or their poor effects *in vivo* of the compounds analyzed o date.

In the recent years small molecules directly targeting the MYC/MAX interaction have been identified. In **paper I**, we have shown that directly targeting MYCN leads to apoptosis, differentiation and lipid accumulation [173, 219, 221].These findings emphasize the possibility of targeting MYC directly. Furthermore, we could see a correlation between decreased protein levels of MYCN in MNA NB cells and lipid accumulation. Our group has previously shown this metabolic reprogramming in response to 10058-F4 which we now extend also to other molecules. Improved MYC inhibitors could be used for cancer therapy in future. Two different approaches could lead to this goal either by improving existing small molecules or by new screening approaches (**paper II**) for improved compounds that might lead to more efficient and specific inhibitors for MYC/MAX interaction. In **paper II** a robust cell-based protein complementary assay was developed for this task showing the success of such an approach. In both **paper I** and **paper II** small molecules have been shown to be more potent in MNA NB cells than in non-amplified NB in inducing apoptosis and inhibition cell growth indicating their selectivity towards MYCN. Promising molecules could be further developed and tested in animal models for their efficiency.

In **paper III** MYCN mediated inhibition of differentiation through up-regulation of miRNAs, miR-18a and miR-19a leading to repression of *ESR1* has been demonstrated. The miR-17~92 cluster has been described having oncogenic functions in cancers such as NB, B-cell lymphoma and anaplastic thyroid cancer. Binding of c-MYC and/or MYCN to the promoters followed by

activation has been reported for several other miRNAs including miR-9, miR-181a, miR-15b, miR-130a as well as miR-214 [258, 259]. These miRNAs might be targeted in two different ways. One possibility would be to target MYC/MYCN in order to inhibit downstream targets including miRNAs, which could also be specifically inhibited by so called anti-miRs [205].

Another strategy is the possibility of targeting downstream targets of MYC or MYC regulated miRNAs. Our group has demonstrated that MYCN regulates several members of the respiratory chain. These members have previously been shown to contribute to metastasis and are correlated with poor survival [165]. Metformin, used for treatment of diabetes, has been proposed to exert anti-tumorigenic activities through inhibition of complex I the respiratory chain [260]. Furthermore it has been proposed to target miRNA-regulated processes such as dedifferentiation in cancer cells [261]. Consequently, treatment with metformin of *MYCN*-amplified and non-*MYCN* amplified NB would be an interesting new approach for future therapy.

Previous effort has been focused on the C-terminal domain bHLHZip of c-MYC, which has been crystallized in a heterodimer with MAX bound to DNA. This domain is known to form two α-helices connected by a loop upon binding to MAX. In **paper IV** we have analyzed the c-MYC-TAD using SAXS showing an overall extended, flexible structure. Some structure was also determined using CD and SAXS analysis indicated the presence of multiple structured domains connected by flexible linkers. SAXS has emerged as an interesting method for characterizing intrinsically disordered proteins.

Importantly, the c-MYC-TAD has been shown to interact with a large amount of different proteins [75, 78]. Molecular basis underlying these interactions are in most cases unclear since this domain is highly flexible an only displays very little secondary structural content. The c-MYC-TAD is necessary for all of MYC's function [58]. Structural and biophysical characterization of the c-MYC-TAD alone as well as in complex with its binding partners will open new possibilities e.g. finding inhibitors for different protein-protein interactions. Specific inhibition of interactions between the c-MYC-TAD and its binding partners might also shed new light on the biological functions of these particular complexes are involved in. Furthermore, this might be an additional approach of targeting MYC in specific types of cancer. TRRAP binds to the c-MYC-TAD and has been reported to inhibit the differentiation of brain tumor initiating cells in glioblastoma multiforme [106]. Splicing forms of another interaction partner BIN-1, normally inhibiting MYC function have been suggested to play a role in tumorigenesis. This might be the result of loss of interaction between the c-MYC-TAD and BIN-1. To date little is known of the molecular basis underlying binding of the c-MYC-TAD and its binding partners. SAXS, NMR as well as SPR and CD could be used to study these interactions between both the wt and mutant forms of c-MYC-TAD and its interactors. Further insights might lead to new approaches for further analyzing MYC function as well as for treatment in certain types of human cancers.

In **paper IV** we have analyzed the c-MYC-TAD using SAXS showing an overall extended, flexible structure. Some structure was also determined using CD and SAXS analysis indicated the presence of multiple structured domains connected by flexible linkers. SAXS has emerged as an interesting method for characterizing intrinsically disordered proteins. In combination with NMR, the possibility arises for determination of structures for disordered and flexible proteins, which has not been possible before. These methods could be used to obtain overall structures of the c-MYC-TAD bound to binding partners and to further characterize these interactions.

In addition, structural insights of the c-MYC-TAD might also be used for structural and functional comparison of different transactivation domains in general. This may lead to common or exclusive features for different transcription factors, which might result in new insights concerning their functions. SAXS has emerged as an interesting method for characterizing intrinsically disordered proteins. In combination with NMR, the possibility arises for determination of structures for disordered and flexible proteins, which has not been possible before. These methods could be used to obtain overall structures of the c-MYC-TAD bound to binding partners and to further characterize these interactions.

In conclusion, different ways of targeting MYC/MYCN have been described in this thesis. This could either be achieved by targeting the interaction of MYC/MYCN with MAX using small molecules or by targeting MYCN-regulated miRNAs in NB. Furthermore, structural characterization of the c-MYC-TAD might lead to the possibility of targeting specific interactions between this domain and one of MYCs binding partners. All these possibilities can be considered as future strategies to target human cancers.

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8. PUBLICATIONS AND MANUSCRIPTS